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THE ROLE OF PHOSPHATIDIC ACID IN  
ASTROCYTE INTRACELLULAR SIGNALLING

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## ABSTRACT

1. Astrocyte-enriched cultures prepared from the neonatal rat cerebral cortex were used to assess the effects of putative mitogens on astroglial proliferation. Subconfluent cultures, 11 div, were deprived of serum, thus halting cell division, and then exposed to these agents. 10% v/v foetal calf serum elicited increases in [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA ranging from 245% to 434% over control. Insulin elicited a dose-dependent increase in [ $^3\text{H}$ ]thymidine incorporation ( $\text{EC}_{50} = 16\mu\text{Uml}^{-1}$ ). Platelet-derived growth factor and phosphatidic acid had a biphasic effect on [ $^3\text{H}$ ]thymidine incorporation apparently due to the presence of high concentrations of their respective vehicles, ethanediol and  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v), at the higher mitogen concentrations tested.

2. The effects of platelet-derived growth factor and phosphatidic acid on [ $^3\text{H}$ ]thymidine incorporation were dependent upon the age of the cultures used. For example, at 5 div platelet-derived growth factor reduced incorporation whereas phosphatidic acid increased incorporation compared to control.

3. The phosphatidic acid mitogenic signal could be amplified from a 13% to 43% increase over control by increasing the time of serum depletion from 24h to 48h. The mitogenic effect of phosphatidic acid was dependent upon the presence of long chain fatty acids.

4. Prolonged phorbol ester pretreatment, abolished the mitogenic action of phosphatidic acid on astrocytes and also reduced that of 10% v/v foetal calf serum by 41%.

5. In order to determine whether the mitogenic effects of phosphatidic acid involved phosphoinositide turnover cultures prelabelled with [ $^3\text{H}$ ]inositol were exposed to phosphatidic acid and inositol phosphates accumulation and inositol phospholipid labelling monitored. Phosphatidic acid elicited a dose- and time-dependent increase in the accumulation of total and individual labelled inositol phosphates which was dependent upon a low extracellular calcium concentration and phosphatidic acid containing long chain fatty acids.

6. Phosphatidic acid stimulated changes in inositol phospholipid labelling and  $^{45}\text{Ca}^{++}$  efflux and both effects were dependent on phosphatidic acid containing long chain fatty acids.

7. Phosphatidic acid had no detectable effect on phosphoinositide turnover but did stimulate  $^{45}\text{Ca}^{++}$  efflux from fibroblasts.

8. The possible release of phosphatidic acid from agonist-stimulated astrocytes was studied by exposing [ $^3\text{H}$ ]arachidonic acid prelabelled cultures to carbachol, ATP and noradrenaline. All three agonists increased the formation of labelled phosphatidic acid but they did not evoke phosphatidic acid release from astrocytes.

9. R59 022, a diacylglycerol kinase inhibitor, inhibited carbachol-stimulated phosphatidic acid production and  $^{45}\text{Ca}^{++}$  efflux but had no effect on carbachol-induced phosphoinositide turnover.

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# ABBREVIATIONS USED.

[ <sup>3</sup> H]AA	[ <sup>3</sup> H]arachidonic acid
AGF 1	astroglial growth factor 1
AGF 2	astroglial growth factor 2
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
Ca <sup>++</sup>	calcium
[Ca <sup>++</sup> ] <sub>i</sub>	intracellular calcium concentration
CBGF	chick brain-derived growth factor
CHCl <sub>3</sub>	chloroform
CNS	central nervous system
DG	diacylglycerol
div	days in vitro
dpm	disintegrations per minute
EGTA	ethyleneglycol-bis-(b-aminoethyl ether)N,N'-tetra acetic acid
EGF	epidermal growth factor
FCS	foetal calf serum
FGF	fibroblast growth factor
GABA	gamma amino butyric acid
GFAP	glial fibrillary acidic protein
GGF	glial growth factor
GM	growth medium
GM-FCS	growth medium without foetal calf serum
GMF	glial maturation factor
GPI	glycerophosphoinositol
GSF	glial stimulating factor
hPDMF	human placental-derived mitogenic factor
IGF I	insulin-like growth factor I

IGF II	insulin-like growth factor II
IL 1	interleukin 1
IL 2	interleukin 2
InsP <sub>1</sub>	inositol monophosphate
InsP <sub>2</sub>	inositol bisphosphate
InsP <sub>3</sub>	inositol trisphosphate
IPs	inositol phosphates
InsP <sub>1</sub>	inositol 1-phosphate
Ins(1,4)P <sub>2</sub>	inositol 1,4-bisphosphate
Ins(1,4,5)P <sub>3</sub>	inositol 1,4,5-trisphosphate
Ins(1,3,4,5)P <sub>4</sub>	inositol 1,3,4,5-tetrakisphosphate
InsP <sub>5</sub>	inositol pentakisphosphate
InsP <sub>6</sub>	inositol hexakisphosphate
MeOH	methanol
NGF	nerve growth factor
O-2A	oligodendrocyte-Type 2 astrocyte
PA	sodium salt of L-phosphatidic acid from egg yolk lecithin. R <sub>1</sub> = C <sub>16/18</sub> , R <sub>2</sub> =arachidonic acid
PA di-C <sub>12:0</sub>	sodium salt of L-phosphatidic acid dilauryl
PA di-C <sub>14:0</sub>	sodium salt of L-phosphatidic acid dimyristoyl
PA di-C <sub>16:0</sub>	sodium salt of L-phosphatidic acid dipalmitoyl
PA di-C <sub>18:1</sub>	sodium salt of L-phosphatidic acid dioleoyl
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PGF <sub>2</sub>	prostaglandin F <sub>2α</sub>
pH <sub>i</sub>	intracellular pH

PMA	phorbol 12-myristate-13-acetate
PtdIns	phosphatidylinositol
PtdIns(4)P	phosphatidylinositol 4-phosphate
PtdIns(4,5)P <sub>2</sub>	phosphatidylinositol 4,5-bisphosphate
PL C	phospholipase C
PL D	phospholipase D
PKC	protein kinase C
PPI	phosphoinositide
PS	phosphatidylserine
PSS	physiological salt solution
SD	standard deviation
SEM	standard error of the mean
TCA	trichloroacetic acid
TLC	thin layer chromatography
TPA	12-O-tetradecanoyl-phorbol-13-acetate

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## CHAPTER 1 GENERAL INTRODUCTION

Over the last ten years it has become clear that the communication processes within the central nervous system (CNS) can no longer be thought of as comprising solely the electrically excitable neurons as the signal processors. The CNS also contains non-excitabile cells. The neuroglia constitute the largest group of these non-excitabile cells, and are divided into macroglia and microglia. Microglia, unlike macroglia, are cells of non-neural origin. Macroglia can be further subdivided into the myelin-producing cells, the oligodendrocytes, and the astrocytes. Astrocytes can be classified depending upon their morphology as being either protoplasmic, mainly situated in the grey matter, or fibrous and located in the white matter. Much evidence has now emerged that points to glia, particularly astrocytes adopting a much more active role in CNS functions than was previously thought.

Astrocytes had been thought of as fulfilling a passive function by acting as a physical packing material located between neurons and the blood supply. In this location they contribute to the blood-brain barrier. The complex physical interactions of astrocytes with neurons and the microvasculature had hampered their study in vivo and it is only since the advent of techniques for the preparation, identification and maintenance in culture of pure or enriched populations of astrocytes that the study of the role of astrocytes in CNS function has been made possible. The ability to produce primary cultures of rat cerebral

astrocytes using, for example, the methods of Manthorpe et al (1979) or McCarthy and De Vellis (1980), and the identification of the astrocyte specific intermediate filament protein, glial fibrillary acidic protein (GFAP) (Eng et al 1971) by immunohistochemistry has provided a convenient in vitro model for such study of astrocyte function.

The increased study of this particular cell type has resulted in the proposal of many more dynamic roles for astrocytes. It is now known that astrocytes provide a guidance system for correct axonal elongation to their target sites (Lauder and McCarthy 1986) and provide trophic factors for neuronal support, differentiation and the promotion of local neuritic outgrowth during development (Manthorpe et al 1986).

Astroglial cells are also involved in the induction of the blood-brain barrier. The endothelial cells of brain capillaries are different from those in the rest of the body in having tight junctions which make them impermeable to water-soluble substances. The capillaries in the brain are surrounded by astrocytic processes and it was thought that these formed the physical barrier. It is now known that the endothelial cells form the blood-brain barrier but that endothelial cell tight junction formation appears to be under the influence of astrocyte-derived factors (Arthur et al 1987; Janzer and Raff 1987; Tao-Cheng et al 1987).

The close physical association of astrocytes with neurons,



neuronal processes and the synaptic cleft has suggested that astrocytes may play a part in the process of neurotransmission. It is now widely accepted that the main method of inactivation of amino acid neurotransmitters is by reuptake after release. Astrocytes have been found to be involved in the inactivation at the synapse of the excitatory and inhibitory neurotransmitters glutamate and gamma amino butyric acid (GABA) respectively. Neuronally-released glutamate and GABA are taken up by astroglial high affinity uptake systems (Hertz and Schousboe 1986; Hosli et al 1986) and metabolised to glutamine by the astrocyte specific enzyme glutamine synthetase (Norenberg and Martinez-Hernandez 1979), which removes the toxic metabolite ammonia at the same time. Glutamine can then be used by neurons as the precursor for subsequent glutamate and GABA synthesis . The presence of high affinity uptake systems in primary cultures of astrocytes for catecholamine and serotonin neurotransmitters may also demonstrate the importance of astrocytes in monoamine neurotransmitter inactivation (Kimmelberg 1986).

As well as their role in transmitter inactivation astrocytes have also been found to function in maintaining the ionic environment for the correct functioning of neurons. Excess extracellular  $K^+$  ions produced by neuronal activity cause depolarisation of the glial cell membrane and  $K^+$  ions are taken up by astrocytes (Gardner-Medwin 1985) possibly via  $K^+$  channels localised in particular regions of the astrocyte membrane (Landis 1986). Astrocytes are also involved in the maintenance of the pH environment of neurons since other ion

transport systems also exist in the astrocyte membrane for the coupled transport-exchange of  $\text{Na}^+$  and  $\text{H}^+$  ions as well as for the exchange of  $\text{Cl}^-$  and  $\text{HCO}_3^-$  (Kimelberg and Ransom 1986).

The presence of receptors for a large number of neuroactive substances on astrocytes, for example, glutamatic acid and GABA (Bowman and Kimelberg 1984; Kettenman et al 1988), the amines 5-hydroxytryptamine (Whitaker-Amitia 1988) and noradrenaline (Hirata et al 1983; Cambray-Deakin 1985; Bockaert and Ebersolt 1988) as well as those for a number of neuroactive peptides (Van Calker and Hamprecht 1980; Wilkin and Cholewinski 1988), (for review see Murphy and Pearce 1987) has lent further weight to the idea that astrocytes are indeed capable of acting as targets for signal molecules released by neurons. These astroglial receptors are functional in that they have been found to be coupled to intracellular second messenger systems. A number of neuroactive substances have been found to be linked to the adenylate cyclase and guanylate cyclase signal transduction pathways in astrocytes (for reviews see Van Calker and Hamprecht 1980; Hamprecht 1986), for example, astroglial  $\beta$ -adrenergic receptor activation increases the level of cAMP (McCarthy et al 1985). Astrocyte receptor activation has also been reported to be linked to phosphoinositide (PPI) turnover (Murphy and Pearce 1987; Pearce and Murphy 1988) with the subsequent mobilisation of cytosolic calcium ( $\text{Ca}^{++}$ ) and protein kinase C (PKC) activation (Berridge 1984; Nishizuka 1986).

The physiological relevance of such receptors on astrocytes is an area of intense research. One question to be answered is whether the activation of these astroglial receptors allows the release of substances which in turn could modulate the activity of adjacent neurons or other cell types in the CNS, thus allowing a two-way communication process.

Kainic acid-induced release of the excitatory amino acids glutamic and aspartic acid from rat hippocampus astrocytes has been observed as well as the release of glutamine and the inhibitory neuromodulator taurine (Lehmann and Hansson 1988).  $\beta$ -adrenergic receptor activation also releases taurine from cultures of astrocytes (Shain et al 1986) and  $\text{Ca}^{++}$ -dependent  $\text{K}^{+}$ -stimulated taurine efflux has been demonstrated from rat cerebellar astrocytes (Philibert et al 1988).

Eicosanoid production and release by astrocytes has been demonstrated (Keller et al 1985; Murphy et al 1985; Jeremy et al 1987; Keller et al 1987; Murphy et al 1988) and this has recently been seen in response to  $\text{P}_2$ -purinergic receptor stimulation (Murphy and Pearce 1988b). Astroglial muscarinic cholinergic receptor activation has been found to produce an increase in a lipxygenase metabolite (De George et al 1986b). These arachidonic acid metabolites may influence not only surrounding neurons but the cells of the adjacent microvasculature as well.

Astrocytes are the predominant glycogen store in the CNS

(Phelps 1972) and have been thought of as performing a nutritive role in relation to neurons. Ordinarily the blood supply to the CNS is thought to provide adequate supplies of glucose for all CNS functions but under conditions when the localised glucose supply may be inadequate, for example, during periods of intense neuronal activity, glucose can be released from astrocytes for use by neurons. Changes in extracellular  $K^+$  concentration as well as neurotransmitter levels can signal neuronal activity to the surrounding astrocytes (Pentreath 1982; Cambray-Deakin et al 1988a; 1988b).

The CNS had been thought of as being insulated from the effects of the immune system. However, the ability of astrocytes to act as antigen-presenting cells and as producers of substances which regulate the activities of cells involved in the immune response, for example, primary cultures of astrocytes have been found to produce and release the leukotriene  $C_4$  (Hartung and Toyka 1987), has again highlighted the astrocyte's ability to interact with other cell types (for review see Fierz and Fontana 1986).

A long established characteristic of astrocytes has been their ability to increase in size and number in response to CNS injury, this process being known as reactive gliosis. Proliferating astrocytes are observed during the pre- and postnatal period of life but the number of proliferating cells decreases with increasing age (Korr 1986). This proliferation of astrocytes after CNS trauma has been assumed to be the reason for the lack of regeneration of the

adult mammalian CNS (Lindsay 1986). Whether mature neurons lack the ability to regenerate or the inability to regenerate is due to the intervention of astrocytes either by direct means, by formation of a physical barrier to axonal elongation, a glial scar, or indirect means due to the lack of neuronotrophic factors and guidance cues is unknown. The formation of a glial scar as a physical barrier to axonal elongation to prevent regeneration is now thought to be a very simplistic explanation in view of the much more dynamic role envisaged for astrocytes within the CNS (Reier 1986). The signals involved in initiating the response of reactive gliosis are not known but in view of the importance of post-trauma proliferation of astrocytes in the CNS factors involved in astroglial proliferation certainly warrant investigation.

Thus the aim of this project was to study the proliferation of rat cortical astrocytes in primary cultures in response to the well-characterised growth factors, insulin and platelet-derived growth factor (PDGF) and the less well-characterised mitogen phosphatidic acid (PA) (Chapter 2). Astroglial proliferation was monitored by using [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA and increased DNA content as measures of DNA synthesis.

Some proliferative agents have been reported to utilise PPI turnover to bring about their growth factor action. In Chapter 3 investigations were undertaken to determine whether the growth factor-like action of PA on astrocytes involved PPI breakdown and calcium mobilisation. The

possible involvement of PA as an intracellular signalling molecule in agonist-stimulated PPI metabolism was considered in Chapter 4 by performing experiments employing an inhibitor of PA production.

This thesis contains three results chapters, each with their own introduction, methods and materials, results and discussion sections. A summary of the general conclusions of the results obtained is given in Chapter 5 together with a discussion of the intracellular function of PA in view of the results obtained in this study and the evidence that is accumulating concerning PA as an intracellular messenger molecule.

## CHAPTER 2 THE EFFECT OF VARIOUS SUBSTANCES ON ASTROCYTE PROLIFERATION

### 2.1. INTRODUCTION

The control of neuronal and glial cell division plays an important role in the correct development of the nervous system. The close physical association of neurons and astrocytes in the CNS has prompted the question of whether the development of these two cell types is interdependent. Consequently, studies of the ways in which these two cell types communicate with each other may shed light on the relative importance of one cell type to the correct growth and development of the other (for reviews see Lauder and McCarthy 1986; Manthorpe et al 1986).

A number of different types of communication are involved and these can be broadly grouped into either signals from the extracellular environment or those involving direct cell-cell communication. Agents present in the extracellular environment, whether produced at a distance from the target cell or by cells adjacent to the target cell, can act to induce or inhibit the growth and/or differentiation of that target cell (Manthorpe et al 1986). Such extracellular agents can also act as chemotactic agents involved in cell migration (Bressler et al 1985). Direct cell-cell communication can occur via specialised areas of contact, for example, gap junctions or coated vesicles (Hatten et al 1984). Cells are also capable of recognising other cells due to the presence of specific cell surface

glycoproteins, for example, neuronal cell adhesion molecule and neuron-glia cell adhesion molecule (Bunge and Waksman 1985). These molecules are involved in regulating correct cellular organisation. Components of the extracellular matrix such as laminin, fibronectin, collagens and proteoglycans may also be involved in determining the correct interactions between neuronal and non-neuronal cell types during development (Hatten et al 1985; Kleinman et al 1981).

Much interest has focused on the extracellular agents that have been found to affect cell growth. The term growth factor is used to describe polypeptide hormones that affect cell growth. Much evidence about growth factors has come from work on cultured murine 3T3 cell lines (Rozengurt 1986). Extracellular growth factors bring about their physiological action by binding to specific high affinity receptors on their target cell. There are two broad groups of growth factor receptors. One group of growth factors have been found to activate protein tyrosine kinase located in the cytoplasmic domain of their receptor causing the redistribution and endocytosis of the receptor (for review see Hunter and Cooper 1985). This activation brings about the phosphorylation of the receptor itself and may be accompanied by the phosphorylation of specific proteins which can then function as intracellular mitogenic signals. Examples of growth factor receptors which possess this protein tyrosine kinase activity include the receptors for epidermal growth factor (EGF) (Ushiro and Cohen 1980), insulin (Goldfine 1987), insulin-like growth factor I (IGF



I) (L'Allemain and Poussegur 1986) and PDGF (Ek et al 1982; Bishop 1985; Rozengurt 1986; Yarden et al 1986). The other group of growth factors interact with receptors which do not possess this intrinsic protein tyrosine kinase activity. Members of this group of growth factors include bombesin, bradykinin, vasopressin and  $\alpha$ -thrombin and the receptors for these factors are thought to couple to GTP-binding proteins (L'Allemain et al 1986; Chambard et al 1987). The coupling of these receptors to a GTP-binding protein (Bourne 1986) activates the hydrolysis of the phosphoinositide phosphatidylinositol 4,5-bisphosphate ( $\text{PtdIns}(4,5)\text{P}_2$ ) by phospholipase C (PL C). The resultant mobilisation of intracellular calcium ( $\text{Ca}^{++}_i$ ) and the activation of PK C are thought to bring about the physiological actions of these growth factors (Michell 1982; Whitman and Cantley 1988). PPI hydrolysis has been shown to play an important part in the regulation of cell proliferation (Berridge 1987b; Nishizuka 1988).

The observation that certain growth factors exhibit synergism has supported the idea that they may operate through the separate signal transduction pathways outlined above. Growth factors have been separated into either competence or progression factors based on which pathway they employ (Murayama and Ui 1987b). Bradykinin, fibroblast growth factor (FGF), PDGF, thrombin and vasopressin (Habenicht et al 1981; Berridge et al 1984) have been classified as competence factors since they allow the initiation of DNA synthesis by the progression factors EGF or insulin. Progression factors activate the protein

tyrosine kinase activity of their own receptors (Macara 1985). The signals elicited by growth factors have also been classified by Rozengurt (1986) into two categories: either regulatory signals or obligatory events. The regulatory signals mediate the action of specific growth-promoting agents, whereas obligatory events are those that must take place for DNA synthesis and cell division to occur. PDGF, which has been classified as a competence factor by Murayama and Ui (1987b), can, in contrast to other competence factors, stimulate DNA synthesis and cell division in Swiss 3T3 cells in the absence of any other factor (Rozengurt 1986). PDGF has been reported to stimulate PPI turnover (Habenicht et al 1981) and the PDGF receptor possesses protein tyrosine kinase activity (Ek et al 1982).

Competence factors stimulate a complex array of early biochemical events. The activation of receptors for competence factors leads to the hydrolysis of the inositol lipid  $\text{PtdIns}(4,5)\text{P}_2$  by PL C which yields the two intracellular second messengers, diacylglycerol (DG) and inositol 1,4,5-trisphosphate ( $\text{Ins}(1,4,5)\text{P}_3$ ) (Berridge and Irvine 1984). DG activation of PKC (Nishizuka 1986) leads to phosphorylation and thus activation of the  $\text{Na}^+/\text{H}^+$  antiporter (Moolenaar et al 1984a) which increases the efflux of  $\text{H}^+$  and the influx of  $\text{Na}^+$  causing intracellular alkalinisation (Moolenaar et al 1983). Increased intracellular pH ( $\text{pH}_i$ ) appears to be a necessary step in the initiation of DNA synthesis (Moolenaar 1986). As well as increases in  $\text{H}^+$  and  $\text{Na}^+$  flux across the plasma membrane an

increase is also seen in  $\text{Ca}^{++}$  efflux (Berridge et al 1984). This increase in efflux, which occurs in the absence of extracellular  $\text{Ca}^{++}$ , is thought to result from an increase in the intracellular  $\text{Ca}^{++}$  concentration ( $[\text{Ca}^{++}]_i$ ) (Moolenaar et al 1984b).  $\text{Ca}^{++}$  is mobilised from intracellular stores by the action of  $\text{Ins}(1,4,5)\text{P}_3$  (Streb et al 1983). These increases in  $\text{pH}_i$  and  $[\text{Ca}^{++}]_i$  appear to be obligatory steps in the initiation of DNA synthesis and represent a common sequence of signals in response to mitogenic stimulation (Hesketh et al 1985).

Genes involved in the regulation of the signal transduction pathways that are required for normal cell proliferation are commonly subverted in the oncogenic, cancer-forming process (Bishop 1987). Proto-oncogene products acquire cell transforming properties as a result of mutations that convert proto-oncogenes to oncogenes. Evidence about the signal transduction pathways used by growth factors in the control of normal cell growth has come from the study of such oncogene products (Berridge 1986). Oncogene products can reduce or eliminate a cell's requirements for the extracellular growth factors that are usually required to initiate proliferation, thus leading to uncontrolled growth. Oncogene products may mimick regulatory signals at a number of steps in mitogenic signal transduction pathways. Oncogene products have been found to be homologous to growth factors and growth factor receptors, for example, the product of the v-sis oncogene is homologous to the B chain of PDGF (Waterfield et al 1983) and that of the v-erb B oncogene is a truncated EGF receptor (Downward et al 1984).

The abl, ros and src oncogene products have been found to have protein tyrosine kinase activity (Hunter and Cooper 1985; Bishop 1985) and that of ras exhibits GTPase activity (McGrath et al 1984). The myc and fos oncogenes are thought to code for proteins which function within the nucleus (Kelly et al 1983; Kruijer et al 1984). The effect of oncogene transformation on PPI turnover has provided evidence for an association between mitogenesis and PPI metabolism. For example, microinjection of transforming protein H-ras into Xenopus oocytes affects the enzymes of PPI breakdown whereas the normal protein has no detectable effect (Lacal et al 1987). All these different stages in the signal transduction pathway obviously have an important part to play in the control of normal cell growth.

Astrocytes have been shown to produce neuronotrophic agents, that is, agents that are required for the growth and survival of neurons, as well as agents that stimulate neuritic extension, neurite promoting factors (Manthorpe et al 1986). Astroglial cells grown in culture have been reported to produce a trophic factor which is involved in the expression of choline acetyltransferase activity by cholinergic neurons from the septal-diagonal band brain region of the 17 day old embryonic rat (Seaton et al 1988). They have also been reported to be a source of brain-derived basic fibroblast growth factor (bFGF) (Ferrara et al 1988) which has been shown to support the survival and elaboration of neurites of cerebral cortical neurons (Morrison et al 1986).

Astroglia are also involved in determining the structural organisation of the brain. During embryogenesis radial glia provide the structural framework for the migration of granule cells in the cerebellum (Mason et al 1988). At later periods in brain development astroglia secrete extracellular matrix components which are thought to promote selective axonal growth (Bunge and Waksman 1985).

As well as a role in neuronal survival and growth during development, astroglial cells are also involved in the response of the CNS to injury. At the site of injury an increase in the size and number of astrocytes occurs leading to the formation of a glial scar. This gliosis is accompanied by a change in astrocyte morphology from the polygonal protoplasmic astrocyte to the fibrous appearance of the reactive astrocyte (Nathaniel and Nathaniel 1981). Following injury the axons of the adult mammalian CNS do not regenerate well, if at all. Astrocytes may be responsible for this lack of regeneration by their proliferation to form a glial scar which then acts as a physical barrier to neurite growth (Nieto-Sampedro et al 1985; Lindsay 1986). Axonal and myelin degradation may also have a role to play in the control of glial proliferation (Politis and Miller 1988). The stimuli involved in switching on this reactive gliosis are unknown. However, the presence of EGF, FGF, insulin, IGF I, insulin-like growth factor II (IGF II), nerve growth factor (NGF) and PDGF, in the brain (Herschmann 1986) has prompted questions about their physiological role in the development, maintenance and function of the CNS. Further evidence of a role for growth factor action in the

CNS comes from in vivo brain injury studies in which increased EGF receptor immunoreactivity has been seen in reactive astrocytes adjacent to the site of injury (Nieto-Sampedro et al). Another interesting question is whether these growth factors could also act as regulators of astroglial function, particularly in reactive gliosis.

The action of a variety of agents on astrocyte proliferation has already been investigated and a summary of these results is presented in Table 2.1. Growth factors which have been shown to increase the proliferation or differentiation of astroglial cells include astroglial growth factor 1 and 2 (AGF 1 and 2), chick brain-derived growth factor (CBGF), foetal calf serum (FCS), glial growth factor (GGF), glial maturation factor (GMF), glial stimulating factor (GSF), IGF I and II, interleukin 1 (IL 1) and human placental-derived mitogenic factor (hPDMF). Contradictory results have been obtained for the effects of EGF, FGF, insulin and PDGF on astroglial proliferation. Differences in the astroglial proliferative response may be dependent on the type of cell preparation investigated. Other agents reported to stimulate proliferation in astroglial cells are carbachol, fibronectin, the ganglioside GM 1, hydrocortisone, the phorbol ester phorbol 12-myristate 13-acetate (PMA), the phospholipid PA, the prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ), putrescine and thrombin. Bradykinin, dibutyryl cyclic AMP, interleukin 2 (IL 2), myelin basic protein, NGF and vasopressin were shown to have no effect on astroglial proliferation. Collagen I and IV, glial-conditioned medium and heparin were found to have inhibitory effects on the proliferation of

Table 2.1. The effects of various agents on astroglial cell proliferation.

Agent	Cell Type			
	Foetal brain cells	Astroglial cells *	Cerebellar astrocytes	Cerebral astrocytes
AGF 1				+28
AGF 2				+28
CBGF				+7
EGF		-34	+18 -15	+3,32 -29
FGF		+9 -34	+15 -18	4,21,22, 23,27,28,29
GGF		+34	+6,14,16	
GMF		+13,19		
GSF		+10		
Insulin	+17	+31	-15	21,22,24 -3,29
IGF I	+17	+31		
IGF II	+17			
NGF		-34		
PDGF		+26,34	-15	+4
hPDMF			+20	
FCS			+15	+3,8,24,33
IL 1				+11,25
IL 2		-34		

Table 2.1. continued.

Agent	Cell Type			
	Foetal brain cells	Astroglial cells *	Cerebellar astrocytes	Cerebral astrocytes
Brady- kinin				- <sup>8</sup>
Carbachol		+ <sup>2</sup>		
Collagen I & IV				x <sup>12</sup>
dibutyryl cAMP		- <sup>34</sup>		
Fibro- nectin				+ <sup>12</sup>
Glial condi- tioned medium			x <sup>1</sup>	
GM 1				+ <sup>33</sup>
Heparin				x <sup>30</sup>
Hydrocor- tisone				+ <sup>21,22</sup>
Myelin basic protein				
PMA		+ <sup>5,34</sup>		+ <sup>24</sup>
PA				+ <sup>23</sup>
PGF2 $\alpha$				+ <sup>21,22</sup>
Putre- scine				+ <sup>21,22</sup>
Thrombin				+ <sup>27</sup>
Vaso- pressin				- <sup>8</sup>

\* Astroglial cells refers to astrocytes obtained from corpus callosum or whole brain. + stimulates proliferation, - no effect on proliferation, x inhibits proliferation.



References for Table 2.1.

1. Aloisi et al 1987
2. Ashkenazi et al 1989
3. Avola et al 1988
4. Besnard et al 1987
5. Bhat 1989
6. Brockes et al 1980
7. Carlone et al 1988
8. Cholewinski and Wilkin 1988
9. Ferrara et al 1988
10. Fontana et al 1981
11. Giulian et al 1988
12. Goetschy et al 1987
13. Kato et al 1979
14. Kim et al 1983
15. Kniss and Burry 1988
16. Lemke and Brockes 1983
17. Lenoir and Honegger 1983
18. Leutz and Schachner 1981
19. Lim and Miller 1984
20. Mercanti et al 1987
21. Morrison and de Vellis 1981
22. Morrison et al 1985
23. Murphy and Pearce 1988a
24. Murphy et al 1987
25. Nieto-Sampedro and Berman 1987
26. Noble et al 1988
27. Perraud et al 1987
28. Pettmann et al 1985

29. Pruss et al 1982
30. Robertson and Goldstein 1988
31. Shemer et al 1987
32. Simpson et al 1982
33. Skaper and Varon 1987
34. Yong et al 1988

astroglial cells. The proliferation of astroglial cells therefore seems to be subject to regulation by a very wide variety of mitogenic agents.

The effects of some of the growth promoting agents outlined in Table 2.1. on neuronal cells as well as non-neuronal cells have also been studied and differences in their effects on these two cell types are seen. Although conflicting reports appear in the literature about the effect of EGF and FGF on astroglial proliferation, EGF has been shown to have no effect on oligodendrocyte or neuronal proliferation (Leutz and Schachner 1981) but FGF has been reported to stimulate both oligodendrocyte and neuronal proliferation (Morrison et al 1986; Perraud et al 1987). GGF has been shown to be an astroglial but not an oligodendrocyte mitogen (Brookes et al 1980) and hPDMF acts on astrocytes but not neurons (Mercanti et al 1987). PDGF's mitogenic activity also extends to oligodendrocytes (Besnard et al 1987) but it does not appear to have any effect on neuronal proliferation (Morrison et al 1986; Besnard et al 1987). Thrombin has been identified as a potent astrocyte mitogen but oligodendrocytes and neurons are unresponsive (Morrison et al 1986; Perraud et al 1987). More than a single growth factor is obviously involved in the growth and development of the CNS but little is yet known about the importance of such differential effects of these growth promoting agents on non-neuronal and neuronal cells.

Interplay also occurs between different cell types in the CNS. Forebrain neurons were found to enhance the

differentiation of astrocytes from different brain regions to different extents. This effect was found to be dependent on both trophic factors (Hunt et al 1987) and direct contact with the nerve cell matrix (Hayashi et al 1988). However, in another study (Hatten and Selanski 1988) cerebellar granule neurons were found to inhibit the proliferation of astrocytoma and cerebellar astroglial cells. Brain fibroblasts have also been found to enhance glial cell (Estin and Vernadakis 1986) and neuroblast proliferation (Gensburger et al 1986). The relative importance of these interactions between different cell types in the CNS is unknown but all contribute to the correct growth and development of the CNS.

The signal transduction mechanisms utilised by the growth factors reported to affect astroglial proliferation are now beginning to be studied, but more information is available from experiments performed on other types of cells. As can be seen from Table 2.2. EGF, FGF, insulin, IGF I and PDGF have all been found to activate protein tyrosine kinase in a variety of different cell types, including fibroblasts and glia. Contradictory results have been obtained for the effect of EGF and insulin on PPI turnover perhaps indicating the existence of receptor subtypes that are linked to different signal transduction pathways in different cells. EGF stimulated PPI turnover in A431 human carcinoma cells but had no effect on 3T3 fibroblasts. Insulin had no effect on PPI turnover in isolated fat cells and BC3H-1 myocytes but DG production and PKC activation have been implicated in its action on BC3H-1 myocytes. Activation of PPI turnover

Table 2.2. The mechanism of action of a number of growth promoting agents

Agent	Kinase activation		Phosphoinositide
	Tyrosine kinase	Protein kinase C	turnover
EGF	+ <sup>12,28,29</sup>	+ <sup>18,19</sup> - <sup>3,13</sup>	- <sup>3,13,15</sup> + <sup>11</sup>
FGF	+ <sup>5</sup>	- <sup>17</sup>	- <sup>17,23</sup>
Insulin	+ <sup>12,14,29</sup>	- <sup>18,27</sup> + <sup>7</sup>	- <sup>24,27</sup> + <sup>9</sup>
IGF I	+ <sup>12,26,29</sup>	?	?
PDGF	+ <sup>8,12,29</sup>	+ <sup>18,19</sup>	+ <sup>10</sup>
Phorbol ester	+ <sup>25</sup>	+ <sup>4,18,20</sup>	- <sup>10</sup>
PA	?	+ <sup>21</sup>	+ <sup>21</sup>
Serum	?	+ <sup>18,19</sup>	+ <sup>15,21</sup>
Bradykinin	?	+ <sup>22</sup>	+ <sup>1,2,6,11</sup>
Thrombin	?	+ <sup>16,18</sup>	+ <sup>1,2</sup>
Vasopressin	?	+ <sup>13,18</sup>	+ <sup>1,2,6</sup>

+ activation, - no activation, ? response not known

References for Table 2.2.

1. Abdel-Latif 1986
2. Berridge and Irvine 1984
3. Blackshear et al 1987
4. Castagna et al 1982
5. Chambard et al 1987
6. Cholewinski and Wilkin 1988
7. Cooper et al 1987
8. Ek et al 1982
9. Farese et al 1986
10. Habenicht et al 1981
11. Hepler et al 1987
12. Herschmann 1986
13. Hesketh et al 1985
14. Kasuga et al 1982
15. L'Allemain and Pouyssegur 1986
16. L'Allemain et al 1986
17. Magnaldo et al 1986
18. Moolenaar 1986
19. Moolenaar et al 1983
20. Moolenaar et al 1984a
21. Moolenaar et al 1986
22. Owen and Villereal 1983
23. Paris et al 1987
24. Pennington and Martin 1985
25. Pierre et al 1986
26. Shemer et al 1987
27. Spach et al 1986
28. Ushiro and Cohen 1980
29. Yarden et al 1986

is not seen in response to FGF. Whereas bradykinin, PA, PDGF, serum, thrombin and vasopressin have been reported to activate PPI turnover. Studies on astrocyte cultures have shown that bradykinin and vasopressin (Cholewinski and Wilkin 1988) as well as PA (Murphy and Pearce 1988a) induce PPI turnover. EGF and insulin have no effect on PPI turnover in astrocytoma cells or astrocytes (Blackshear et al 1987; Pearce et al unpubl.).

Primary cultures of rat neonatal cortical astrocytes provide a useful model for the investigation of the proliferative action of various agents. A cell population enriched in astroglial cells is obtained by mechanical and enzymatic dissociation of rat neonatal cerebral cortices. Such primary cultures produce sufficient material to allow biochemical investigations to be carried out. Using the cell culture method outlined in the Methods and Materials section of this chapter confluent astrocyte-enriched cultures are obtained by 18 days in vitro (div). In order to assess the proliferative action of a number of growth factors, younger cultures, that are still capable of proliferating, are required. Consequently, subconfluent cultures grown for 11 div were used. Astrocytes in vitro are randomly distributed with respect to their position in the cell cycle. Synchronisation of these cells allows an amplification of the response to specific growth factors because a larger proportion of the cells are then in the same phase of the cell cycle. By reducing the serum content of the growth medium a quiescent state can be achieved, where growth of the cells is arrested but they are still

viable. The cells can then be stimulated to re-enter the cell cycle by re-addition of serum or other growth factors. This synchronisation technique has provided a sensitive method for the identification of glial mitogens (Nieto-Sampedro et al 1985; Langan and Volpe 1986; Murphy et al 1987; Kniss and Burry 1988).

In the remainder of this chapter I shall present evidence to show that:

- (i) Insulin, PDGF and PA stimulated cerebral astrocyte proliferation as measured by [<sup>3</sup>H]thymidine incorporation into DNA.
- (ii) The proliferative action of PDGF and PA differed with the age of cerebral astrocytes in culture.
- (iii) The effect of PA on cerebral astrocyte proliferation was dependent on PA containing long chain fatty acids.
- (iv) Phorbol ester pretreatment abolished the proliferative action of PA and reduced that of FCS on cerebral astrocytes.



## 2.2 METHODS AND MATERIALS

### 2.2.1. Astrocyte Cell Culture.

Astrocyte-enriched primary cultures from neonatal rat cerebral cortex were prepared essentially according to the method of Dutton et al (1981). The brains of 1 or 2 day old rat pups were removed and the cerebral cortices detached from the hind brain and the underlying structures. The cortices were then thoroughly cleaned of meninges and chopped at 400 $\mu$ m intervals with two passes at right angles to each other using a McIlwain tissue chopper. The tissue was enzymatically dissociated by suspension in a trypsin solution (250  $\mu$ gml<sup>-1</sup>) made up in disaggregation medium of the following composition: glucose 14 mM, bovine serum albumin (BSA) 3 mgml<sup>-1</sup> and MgSO<sub>4</sub>.7H<sub>2</sub>O 1.5 mM in Ca<sup>++</sup>- and Mg<sup>++</sup>-free Earles balanced salt solution. The suspension was transferred to a trypsinization flask and incubated in a shaking water bath at 37°C for 15 minutes. A solution containing trypsin inhibitor (192  $\mu$ gml<sup>-1</sup>), DNase (6.4  $\mu$ gml<sup>-1</sup>) and MgSO<sub>4</sub>.7H<sub>2</sub>O (240  $\mu$ M) in disaggregation medium was then added and the resulting suspension transferred to plastic tubes and centrifuged at 200g for 5 seconds. The supernatant was removed and the cell pellet resuspended in a solution containing trypsin inhibitor (1.2 mgml<sup>-1</sup>), DNase (40  $\mu$ gml<sup>-1</sup>) and MgSO<sub>4</sub>.7H<sub>2</sub>O (1.5 mM) in disaggregation medium.

The cells were then mechanically dissociated by trituration through a 1.5mm diameter stainless steel cannula. The

resulting cell suspension was underlaid with a 4% w/v BSA solution and the intact cells were pelleted through the BSA underlay by centrifugation at 200g for 5 minutes. The supernatant and the BSA underlay, which contained cell debris, were removed and the cell pellet resuspended in a small volume of growth medium (GM) of the following composition: Eagles Minimal Essential Medium containing Earles salts (glutamine-free) supplemented with FCS 10% v/v, glutamine 2 mM, glucose 33 mM and gentamycin sulphate 65  $\mu\text{gml}^{-1}$  (10% v/v FCS GM). The number of cells in the suspension was determined by diluting 20  $\mu\text{l}$  of suspension in 20ml Isoton buffer and counting 0.5ml samples of this suspension using a Coulter counter (Coulter Electronics Ltd.) with a 140  $\mu\text{m}$  aperture. The cell suspension was then appropriately diluted with 10% v/v FCS GM to give a seeding density of 100 cells  $\text{mm}^{-2}$ . Cells were then seeded onto poly-D-lysine-coated (50  $\mu\text{gml}^{-1}$ ) 60mm. diameter dishes, 6 well plates (35mm. diameter) or 24 well plates (15mm. diameter). The cells were grown in a humid, 5%  $\text{CO}_2$  / 95% air atmosphere at 37°C and the medium was renewed every 3 or 4 days. Confluent cultures are obtained by 18 div. Subconfluent cultures were used at 11 div.

Comprehensive characterisation of these astrocyte-enriched cultures has been carried out in this laboratory by Cambray-Deakin (Ph.D. thesis, Open University) and Murphy (unpubl.). Using indirect immunofluorescence the prominent cell type in these cultures was found to be labelled by antibodies to the astrocyte specific marker glial fibrillary acidic protein (GFAP). The majority of these GFAP+ cells were of a

flattened, polygonal morphology, that is, Type I astrocytes (Temple and Raff 1985). Very few process-possessing astrocytes, Type II cells, were seen. These cultures were not labelled by antibodies to neurofilament protein or galactocerebroside indicating the absence of neuronal and oligodendroglial cells. Cells labelled with antibodies to fibronectin (FN+) were seen indicating the presence of fibroblasts. These fibroblasts were found to be of meningeal origin and good removal of the meninges was essential to reduce meningeal fibroblast contamination of the astrocyte-enriched cultures. The amount of FN+ cells present did vary from batch to batch of astrocyte-enriched cultures but routinely constituted only 5-10 % of the cells present. To control for the contamination of these astrocyte-enriched cultures by fibroblasts, meningeal fibroblast cultures were prepared (for culture method see Chapter 3 section 3.2.5.). These meningeal cultures contained densely-packed, multi-layered, columnar cells. In some studies their response was compared to that of astrocyte-enriched cultures. The nature of these astrocyte-enriched cultures must be borne in mind when discussing studies using these preparations. However, they do provide a source of a sufficient quantity of an enriched population of astrocytes for biochemical analysis in which the major contaminating cell type has been identified and whose presence can be controlled for.

Examination of the astrocyte-enriched cultures by phase contrast microscopy showed the presence of round, phase bright cells on top of the astrocyte layer. The

phagocytosis of Indian ink by these cells identified them as macrophages. The macrophage content of these cultures also varied, increasing with time in culture, but these macrophages were easily removed from the astrocyte layer by thorough washing.

A sample from each batch of cultures was examined by phase contrast microscopy prior to experimentation to check the state of the astrocyte layer and to estimate whether there was gross fibroblast contamination.

2.2.2. Assessment of astrocyte proliferation in response to a number of growth factors.

2.2.2.1. [ $^3\text{H}$ ]Thymidine incorporation.

The proliferative action of various agents was determined by measuring [ $^3\text{H}$ ]thymidine incorporation into these primary cultures of astrocytes. [ $^3\text{H}$ ]Thymidine is nearly exclusively incorporated into the DNA of cells which are in S phase of the cell cycle, that is, DNA synthesis is occurring at the time of [ $^3\text{H}$ ]thymidine application. DNA turnover in the course of DNA repair also incorporates [ $^3\text{H}$ ]thymidine but the rate of incorporation is much lower than in dividing cells (Korr 1986). [ $^3\text{H}$ ]Thymidine incorporation is therefore interpreted as labelling in the course of DNA synthesis preparing for cell division, that is, labelling of a proliferating cell.

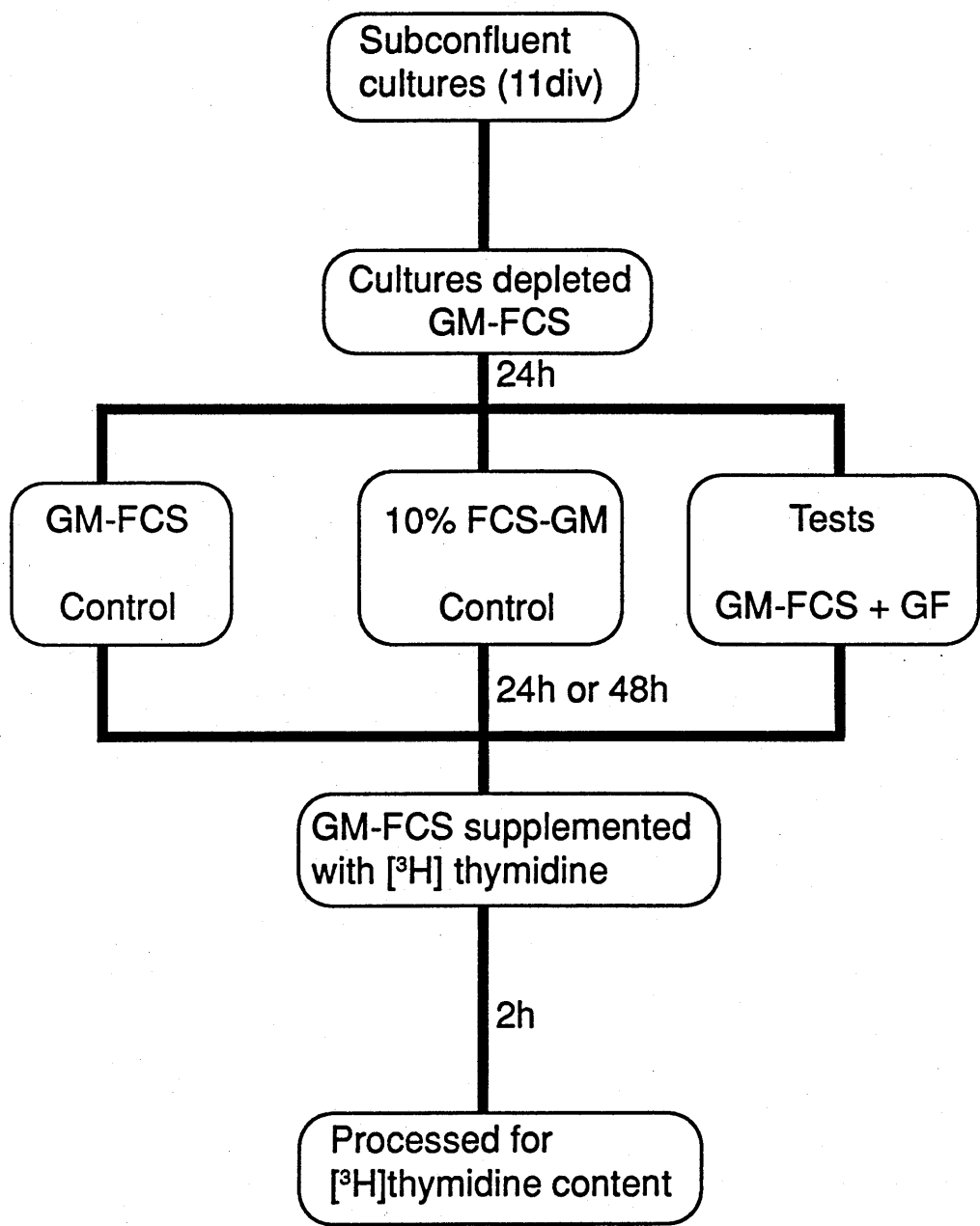
Quiescent astrocytes were obtained by depleting astrocytes

of serum for 24h. The astrocytes were grown in 24 well plates for 11 div. Serum depletion was carried out by rinsing the cultures with growth medium without FCS (GM-FCS) and then incubating them in fresh GM-FCS for 24 hours at 37°C in a 5% CO<sub>2</sub> / 95% air atmosphere. The depletion medium was then removed, the cells rinsed and fresh GM-FCS added to which the appropriate growth factor was added. The cultures were then incubated for 24 or 48h. In each experiment two controls were also performed. After serum depletion some cells had fresh GM-FCS only added to them. These controls were performed to determine the basal level of methyl-[<sup>3</sup>H]thymidine incorporation into DNA in the absence of any added growth factor. Another set of cells had 10% v/v FCS GM added backed to them to assess their response to serum, a potent mitogen for astrocytes. The inclusion of these controls allowed the comparison of results from different batches of astrocytes. A summary of the experimental protocol followed is shown in Figure 2.1.

DNA synthesis by these cultures was then monitored by the incorporation of methyl-[<sup>3</sup>H]thymidine essentially according to the method described by Murphy et al (1987). The exposure medium was removed, the cells rinsed with GM-FCS and fresh GM-FCS supplemented with 1  $\mu$ Ci of methyl-[<sup>3</sup>H]thymidine was added to each well and after 2 hours incubation the labelled medium removed. The cells were washed with physiological salt solution (PSS) of the following composition : NaCl 116 mM, NaHCO<sub>3</sub> 26 mM, glucose 20 mM, KCl 2.5 mM, MgSO<sub>4</sub>.7H<sub>2</sub>O 1.5 mM, NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O 1 mM and phenol red 10 mg/500ml pre-gassed with 5% CO<sub>2</sub> / 95% air.

Figure 2.1. Summary of experimental protocol for assessment of growth factor-induced [<sup>3</sup>H]thymidine incorporation into astrocyte-enriched cultures.

FIG. 2.1



After washing the cells were solubilised in 500 $\mu$ l 0.1M NaOH. The cells were scraped from the wells after 10 to 15 minutes and transferred to eppendorf tubes. BSA (250  $\mu$ g/well) was added as a carrier and protein precipitated with 10% w/v trichloroacetic acid (TCA). After 10 minutes at 4°C, the precipitated protein was pelleted by centrifugation at 1,200g for 10 minutes. After two more washes with TCA the protein pellets were solubilised overnight with 250 $\mu$ l Protosol. The Protosol suspension was then neutralised with 250 $\mu$ l 2M HCl to reduce chemiluminescence and 8ml of the toluene-based scintillant Cocktail T added. After mixing, the [<sup>3</sup>H]thymidine content of each sample was assessed by liquid scintillation counting.

#### 2.2.2.2. DNA content

The proliferative action of PA on astrocyte-enriched cultures was also assessed by measuring the DNA content of treated cultures using the fluorochrome Hoechst 33258, (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol.3HCl) (also known as bis-benzimidazole) essentially according to the method of Labarca and Paigen (1980). This method is simple, rapid and convenient compared to the time-consuming [<sup>3</sup>H]thymidine incorporation technique. Hoechst 33258 binds specifically to DNA. These experiments were carried out essentially as described for the [<sup>3</sup>H]thymidine incorporation experiments, except that cultures were grown in 6 well plates for 10, 11 or 12 div. Serum depletion was carried out by incubation in GM containing 0.1% v/v FCS instead of GM-FCS because longer



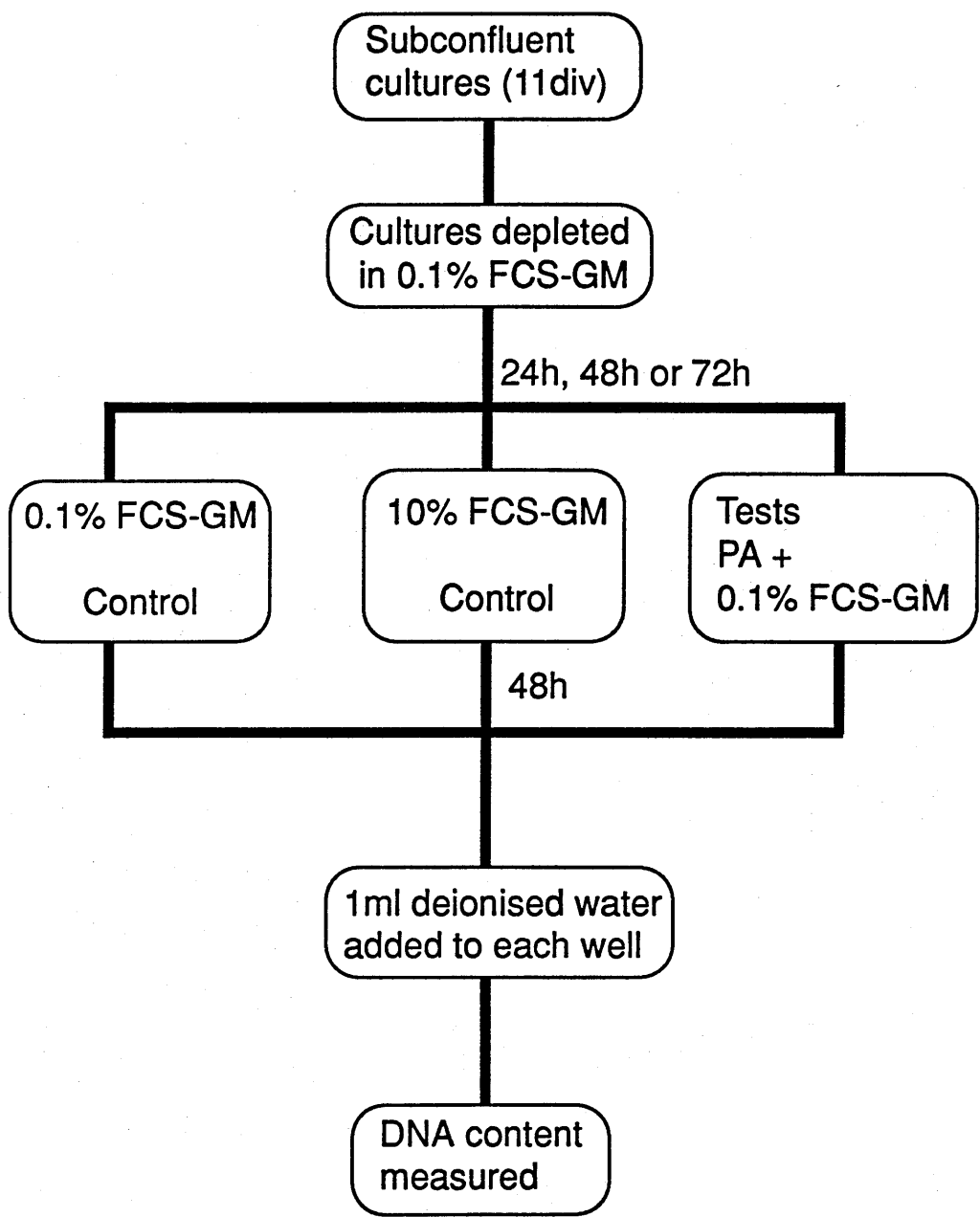
depletion times of 48h and 72h were used as well as 24h.

Cells were then exposed for 48h to either 0.1% v/v FCS GM, 10% v/v FCS GM or 0.1% v/v FCS GM and PA at the appropriate concentration. A summary of the experimental protocol used is shown in Figure 2.2.

After rinsing twice with PSS, 1ml of deionised H<sub>2</sub>O was added to each well. The cells were then frozen and thawed and the DNA content of the resulting cell suspension determined. An aliquot of cell suspension in 1ml of H<sub>2</sub>O was added to 1ml of DNA assay buffer which contained 2  $\mu\text{gml}^{-1}$  Hoechst 33258, 4M NaCl in 0.1M sodium phosphate buffer pH7.4, giving a final Hoechst 33258 concentration of 1  $\mu\text{gml}^{-1}$ . Hoechst 33258 gains access to the DNA once the chromatin has been dissociated by the presence of the high salt concentration and maximum enhancement of fluorescence is seen at pH 7.4. After thorough mixing the fluorescence of the sample was determined using an excitation wavelength of 356nm, an emission wavelength of 492nm and a slit width of 5nm. The fluorescence obtained was compared to that obtained for a standard DNA solution from calf thymus. The fluorescence of a reagent blank containing 1ml H<sub>2</sub>O and 1ml DNA assay buffer was determined. The fluorescence of the challenge compounds and the PSS washes was also checked to see if they interfered with the DNA assay.

Figure 2.2. Summary of experimental protocol for assessment of growth factor action on DNA content of astrocyte-enriched cultures.

FIG. 2.2



### 2.2.2.3. Phorbol ester pretreatment

Tumour-promoting phorbol esters are capable of mimicking some of the effects of DG on PKC (Ashendel 1985). The phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) has been shown to activate PKC directly without activating PPI turnover (Castagna et al 1982). In astrocytes, the proliferative action of the phorbol ester PMA suggests a role for PKC activation in the control of glial cell growth (Murphy et al 1987; Bhat 1989).

The contribution made by PKC activation to the mitogenic action of PA on astrocytes was assessed by treating astrocyte-enriched cultures with the phorbol ester PMA, and comparing the effect of PA on treated and untreated cells. Phorbol ester pretreatment was carried out by incubating subconfluent cultures (11 div) with  $1\mu\text{M}$  PMA in 0.1% v/v FCS GM for 6h essentially according to the method of McCardle et al (1987). After removing the PMA medium, the cells were rinsed and incubated for a further 42h in 0.1% v/v FCS GM. Untreated cultures were incubated in 0.1% v/v FCS GM only for 48h. This PMA pre-treatment has been shown by other workers in this laboratory to reduce astrocyte PKC activity by almost 95% for up to at least 96h after treatment (Murphy and Pearce 1988a). Treated and untreated cells were then exposed for 48h to either 0.1% v/v FCS GM, 10% v/v FCS GM or 0.1% v/v FCS GM and PA at  $100\mu\text{gml}^{-1}$ . After exposure, astrocyte cell suspensions were obtained and the DNA content determined as described above. A summary of the experimental protocol used is shown in

Figure 2.3.

#### 2.2.3. Growth factor additions

The insulin stock, 26.9 units  $\text{ml}^{-1}$  in deionised water and the PDGF stock, 167 units  $\text{ml}^{-1}$  in 1M NaCl, 10 mM  $\text{Na}_2\text{PO}_4$  pH 7.4 in 50% v/v ethanediol were both diluted appropriately in GM-FCS for addition. In initial experiments PA (Sigma) was dissolved in chloroform:methanol (1:2 v/v) ( $\text{CHCl}_3$ :MeOH 1:2 v/v) and this stock diluted appropriately for use.

Subsequent experiments were performed using an aqueous suspension of PA (Sigma or Serdary). An aqueous suspension of PA in PSS was obtained by sonication at  $10\mu\text{m}$  for two 10 second bursts on ice, with a 30 second rest in between, using a Soniprep 150 (MSE Scientific Instruments). A 5 or 10  $\text{mgml}^{-1}$  stock was freshly prepared and appropriately diluted for use.

#### 2.2.4. Statistical analysis

Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01, \*\*\*\* 0.001.

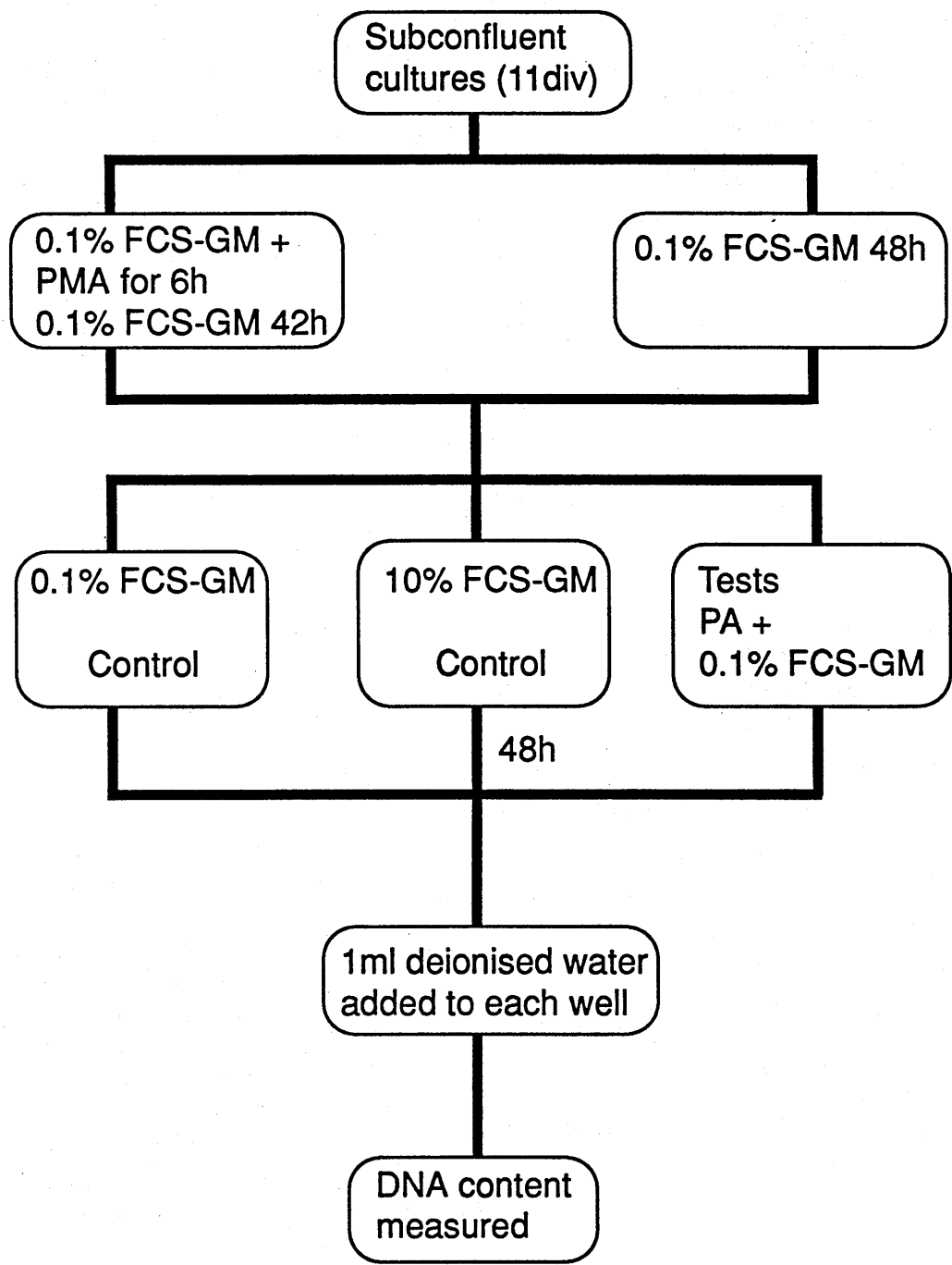
#### 2.2.5. Materials

All materials used for tissue culture were obtained from Gibco Ltd. or Sigma Chemical Co.. Plasticware for tissue culture was obtained from Nunc. Hoechst 33258, insulin, the sodium

salts of all the following PAs: PA from egg yolk lecithin, PA containing lauric acid (PA di-C<sub>12:0</sub>), PA containing myristic acid (PA di-C<sub>14:0</sub>), PA containing palmitic acid (PA di-C<sub>16:0</sub>) and PA containing oleic acid (PA di-C<sub>18:1</sub>) and PMA were obtained from Sigma. PA (NH<sub>4</sub> salt) was obtained from Serdary Research Labs., Canada. Partially purified PDGF from porcine platelets was obtained from Bioprocessing Ltd., County Durham. Protosol, the tissue and gel solubiliser was obtained from New England Nuclear. Cocktail T scintillant and the materials for the incubation medium were obtained from BDH. Methyl[<sup>3</sup>H]thymidine (51Ci/mmol) was obtained from Amersham International PLC.

Figure 2.3. Summary of the experimental protocol for assessment of the contribution made by PKC activation to the growth factor action of PA on astrocytes.

FIG. 2.3





## 2.3. RESULTS

### 2.3.1. The effect of insulin and PDGF on [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA.

Preliminary experiments were carried out using growth factor-induced [ $^3\text{H}$ ]thymidine incorporation into the DNA of subconfluent astrocyte cultures (11 div), as a measure of astrocyte DNA synthesis. Cells were grown in 24 well plates (15mm diameter). In initial experiments the mitogenic action of insulin and PDGF on astrocytes was investigated, since both of these growth factors have been reported to stimulate DNA synthesis in astrocytes (Murphy et al 1987; Besnard et al 1987).

From Fig. 2.4a it can be seen that insulin caused a dose-dependent ( $\text{EC}_{50} = 16 \mu\text{Uml}^{-1}$ ) increase in [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA after a 48h exposure. Maximum stimulation was found at  $1000 \mu\text{Uml}^{-1}$  insulin, and this was equivalent to 74% of the response produced by exposure to GM containing the potent mitogen FCS. 10% FCS GM caused a 345% increase in [ $^3\text{H}$ ]thymidine incorporation compared to the GM-FCS control.

In contrast to the effect of insulin, increasing concentrations of PDGF produced a biphasic dose-response curve for [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA after a 48h exposure (Fig. 2.4b). No increase in [ $^3\text{H}$ ]thymidine incorporation was seen in response to 0.005 and  $0.025 \text{ Uml}^{-1}$  PDGF but 0.050, 0.125, 0.250 and  $0.500 \text{ Uml}^{-1}$

Fig. 2.4a and b. Insulin- and PDGF-induced [<sup>3</sup>H]thymidine incorporation into astrocyte DNA.

Subconfluent astrocyte cultures (11 div) were serum depleted for 24h and then exposed to various concentrations of either insulin or PDGF for 48h. Results are expressed as % of GM-FCS control. Results for insulin (fig. 2.4a) are mean +/- standard error of the mean (SEM) of 6 values, from 2 experiments using 24 well plates. Mean disintegrations per minute (dpm) for GM-FCS control = 706 dpm. Results for PDGF (fig. 2.4b) are mean +/- SEM of values from 4 experiments (n = from 3 to 15) using 24 well plates. Mean dpm for GM-FCS control = 386 dpm. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to appropriate GM-FCS control.

FIG 2.4 A

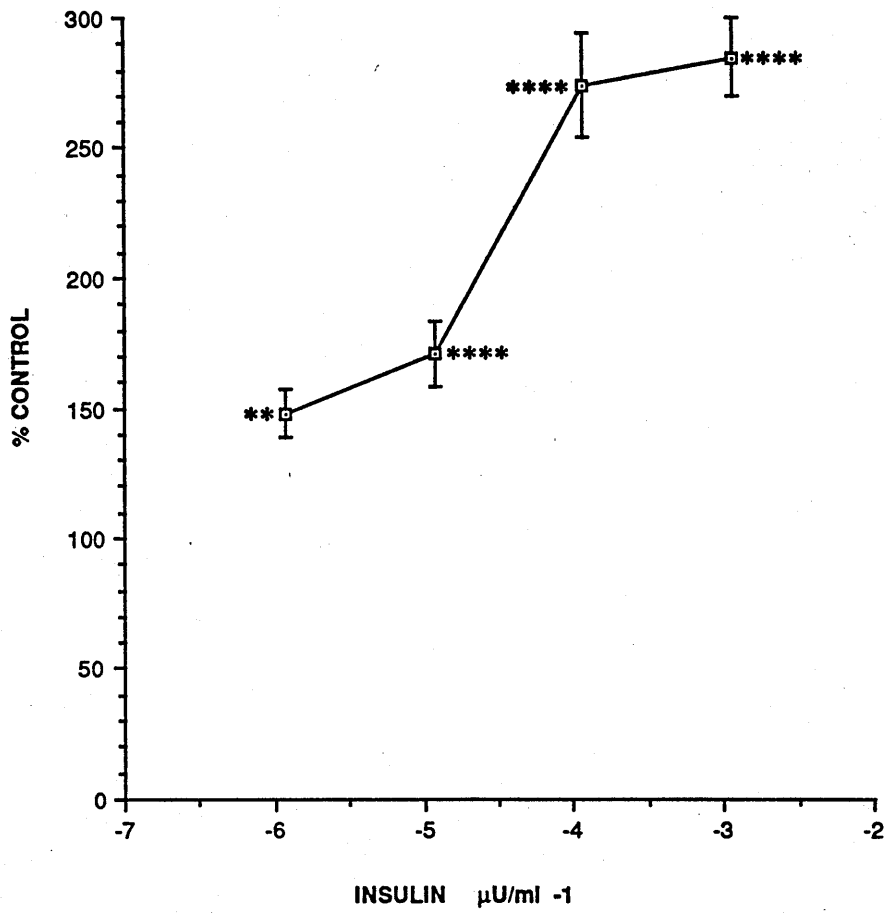
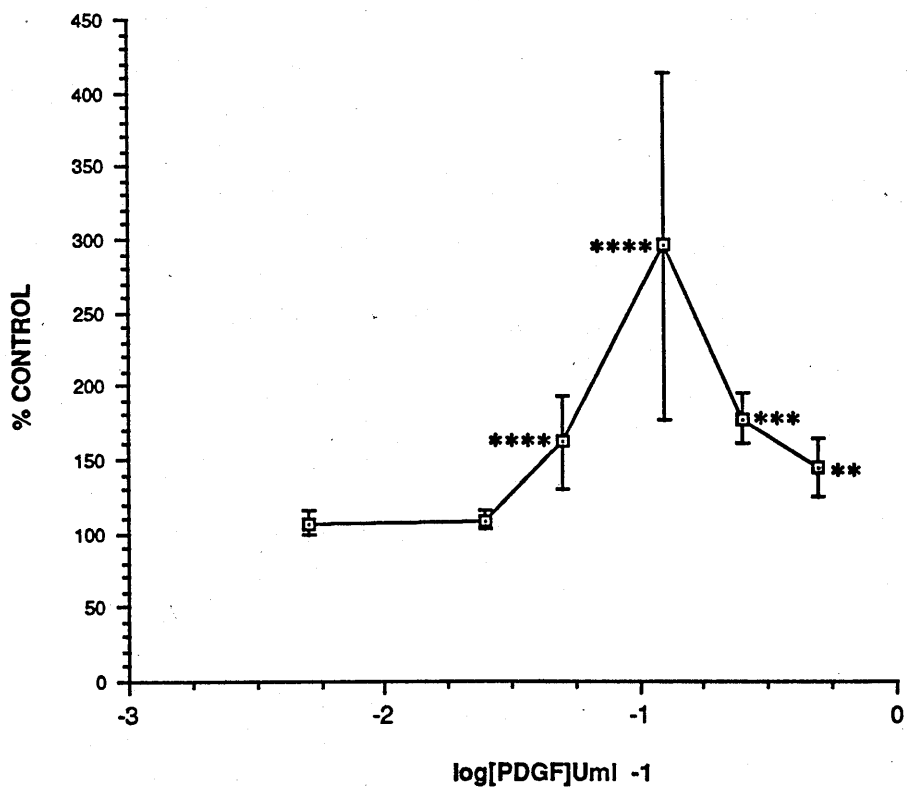


FIG 2.4B



PDGF all caused significant increases in [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA. Maximum stimulation, of 196% over GM-FCS control, was found at  $0.125 \text{ Uml}^{-1}$  PDGF. This response was equivalent to 45% of that elicited by 10% FCS GM in the same experiments.

However, at concentrations of  $0.250$  and  $0.500 \text{ Uml}^{-1}$ , PDGF-stimulated [ $^3\text{H}$ ]thymidine incorporation was lower than that elicited by  $0.125 \text{ Uml}^{-1}$  PDGF. The PDGF preparation used in these experiments was supplied in a solution containing 50% v/v ethanediol as a preservative. In order to determine whether the lower incorporation responses that were seen at the higher PDGF concentrations tested ( $0.250$  and  $0.50 \text{ Uml}^{-1}$ ) were due to the higher final concentrations of ethanediol ( $0.75$  and  $1.50 \%$  v/v respectively), a preliminary experiment was carried out to determine the effect of ethanediol on 10% FCS-stimulated [ $^3\text{H}$ ]thymidine incorporation. The effect of the potent mitogen 10% FCS GM in the presence of increasing concentrations of ethanediol on [ $^3\text{H}$ ]thymidine incorporation was compared to that in the presence of 10% FCS GM alone. A 36% reduction in [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA was seen in response to 10% FCS GM in the presence of 1.5% v/v ethanediol compared to that seen in response to 10% FCS GM alone (Fig. 2.5). 10% FCS GM elicited an increase in DNA synthesis that was 313% greater than that seen in response to the GM-FCS control.

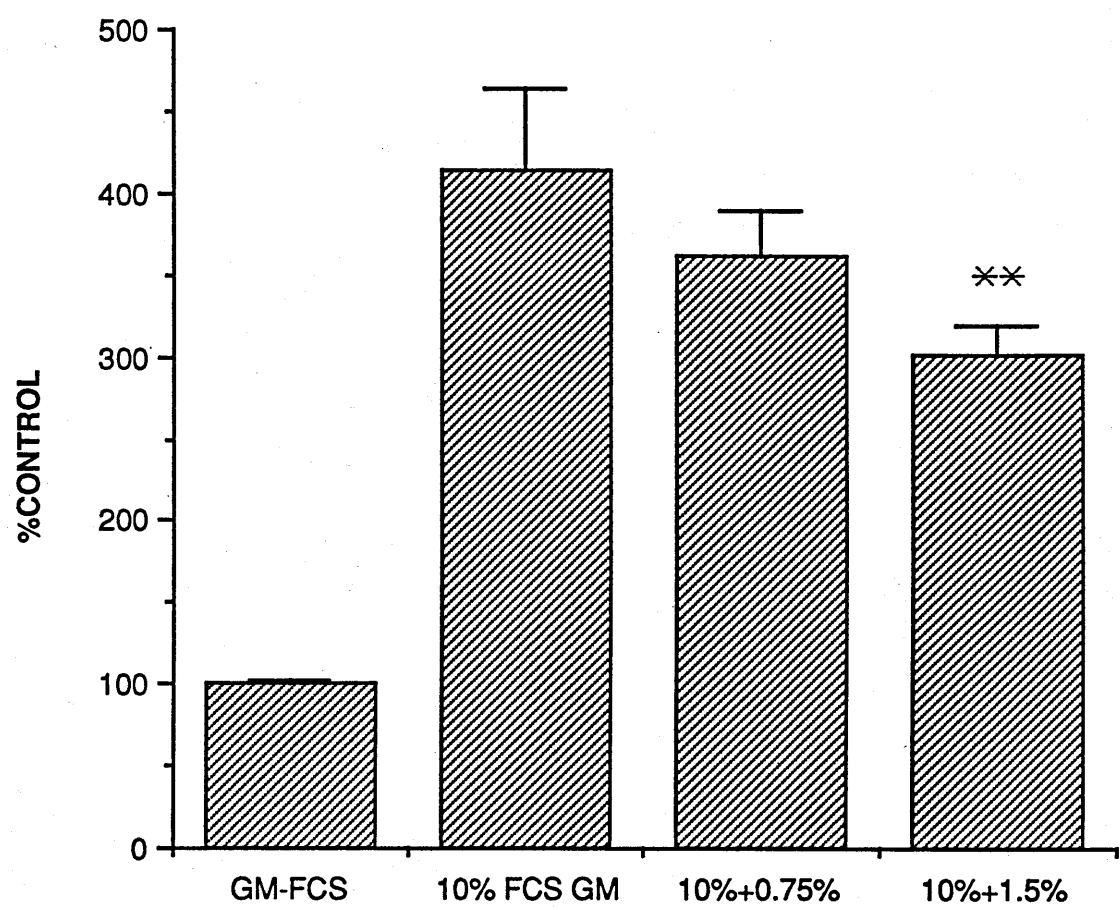
Some variation was seen in the response of different batches of astrocytes to 10% FCS GM. Increases of 245%, 434% and

Fig. 2.5. The effect of increasing ethanediol concentration on 10% FCS GM-induced [<sup>3</sup>H]thymidine incorporation into astrocyte DNA.

Subconfluent cultures (11 div) were serum depleted for 24h and then exposed to either 10% FCS GM alone or 10% FCS GM in the presence of increasing concentrations of ethanediol for 48h. Results are mean +/- SEM for n = 3 values from 1 experiment using 24 well plates. Results are expressed as % of GM-FCS control. GM-FCS control mean dpm = 771.

Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Ethanediol containing samples compared to 10% FCS GM control.

FIG. 2.5



313% over the GM-FCS control were seen in the insulin, PDGF and ethanediol experiments respectively. All these experiments were performed on astrocyte cultures at 11 div but astrocytes grown for the same length of time in vitro may still show different rates of growth. The effect of PDGF on younger astrocyte cultures was therefore investigated to see if there was a variation in the responsiveness of astrocytes to proliferative stimuli with respect to age in culture. The effect of increasing concentrations of PDGF on [<sup>3</sup>H]thymidine incorporation into astrocyte DNA of 5, 6 and 8 div cultures was investigated.

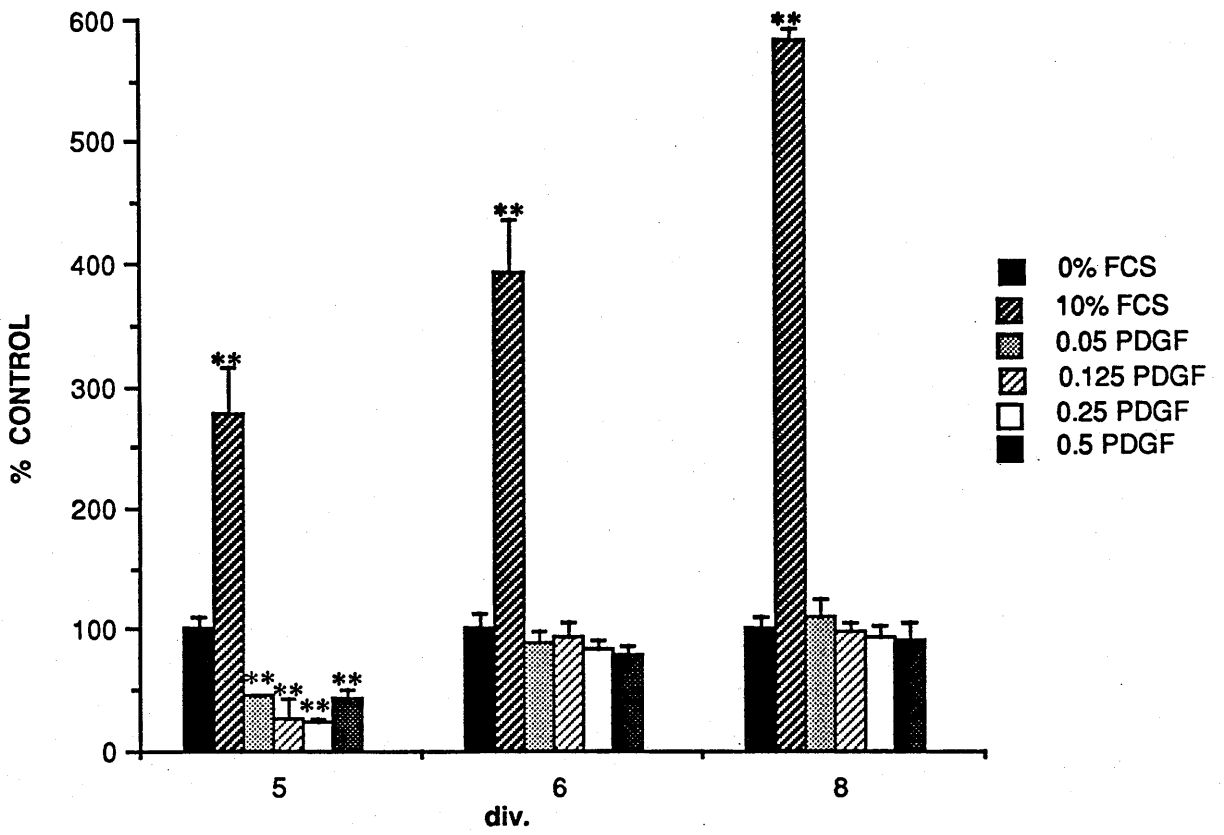
PDGF had no stimulatory effect on incorporation into astrocyte DNA of 6 or 8 div cultures at any of the concentrations tested when compared to the GM-FCS control (Fig. 2.6). PDGF, at all concentrations tested had an inhibitory effect on [<sup>3</sup>H]thymidine incorporation into cells grown for 5 div. Reductions of 55%, 73%, 77% and 56% compared to the GM-FCS control were seen in response to 0.050, 0.125, 0.250 and 0.500 Uml<sup>-1</sup> PDGF respectively. Variation was also found in the response of different age cultures to 10% FCS GM. Cells became more responsive to 10% FCS GM with increasing age in culture. At 5 div an increase of 178% over the GM-FCS control was seen which increased to 292% at 6 div and 483% at 8 div. The 10% FCS GM response in the previous PDGF experiment using 11 div cultures was a 434% increase over the GM-FCS control.

Fig. 2.6. PDGF-induced [<sup>3</sup>H]thymidine incorporation into 5, 6 and 8 div astrocytes.

Astrocyte cultures 5, 6 or 8 div were serum depleted for 24h and then exposed to increasing concentrations of PDGF for 48h. Results are mean +/- SEM for 3 or 4 values from 1 experiment using 24 well plates and are expressed as % of GM-FCS control. GM-FCS control mean dpm = 236 at 5 div, 409 at 6 div and 912 at 8 div. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to appropriate GM-FCS control.



FIG. 2.6



2.3.2. The effect of PA on [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA.

PA obtained from Sigma caused an increase in [ $^3\text{H}$ ]thymidine incorporation into astrocytes when compared to the GM-FCS control after 48h exposure (Fig. 2.7). PA had a significant stimulatory effect at 20 and 50  $\mu\text{gml}^{-1}$  but not at 75 or 100  $\mu\text{gml}^{-1}$ .

For these experiments PA was diluted from a 10  $\text{mgml}^{-1}$  stock made up in  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v). The highest PA concentration tested of 100  $\mu\text{gml}^{-1}$  consequently contained 1% v/v  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v). The lack of response of astrocytes to the higher PA concentrations tested may have been due to a detrimental effect of the  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) present on the astrocytes as was the case for ethanediol in the PDGF experiments. To test this idea the influence of increasing concentrations of  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) on the effect of 10% FCS GM on [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA was investigated. Astrocytes were incubated with 10% FCS GM in the presence of increasing concentrations of  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) for 48h and the response of these cells compared to that in response to 10% FCS GM alone.

The presence of 0.5% and 1.0% v/v  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) had a significant effect on the activity of 10% FCS GM on [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA. When solvent was present at these concentrations lower incorporation was seen when compared to that in the presence of 10% FCS GM alone (Fig.2.8). Reductions of 31% and 46% in

Fig. 2.7. PA-induced [<sup>3</sup>H]thymidine incorporation into astrocyte DNA.

Subconfluent astrocyte cultures (11 div) were serum depleted for 24h and then exposed to PA for 48h. Results are mean +/- SEM for n = 9 values from 3 experiments using 24 well plates and are expressed as % of GM-FCS control. Mean dpm for GM-FCS control = 435. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to GM-FCS control.

FIG. 2.7

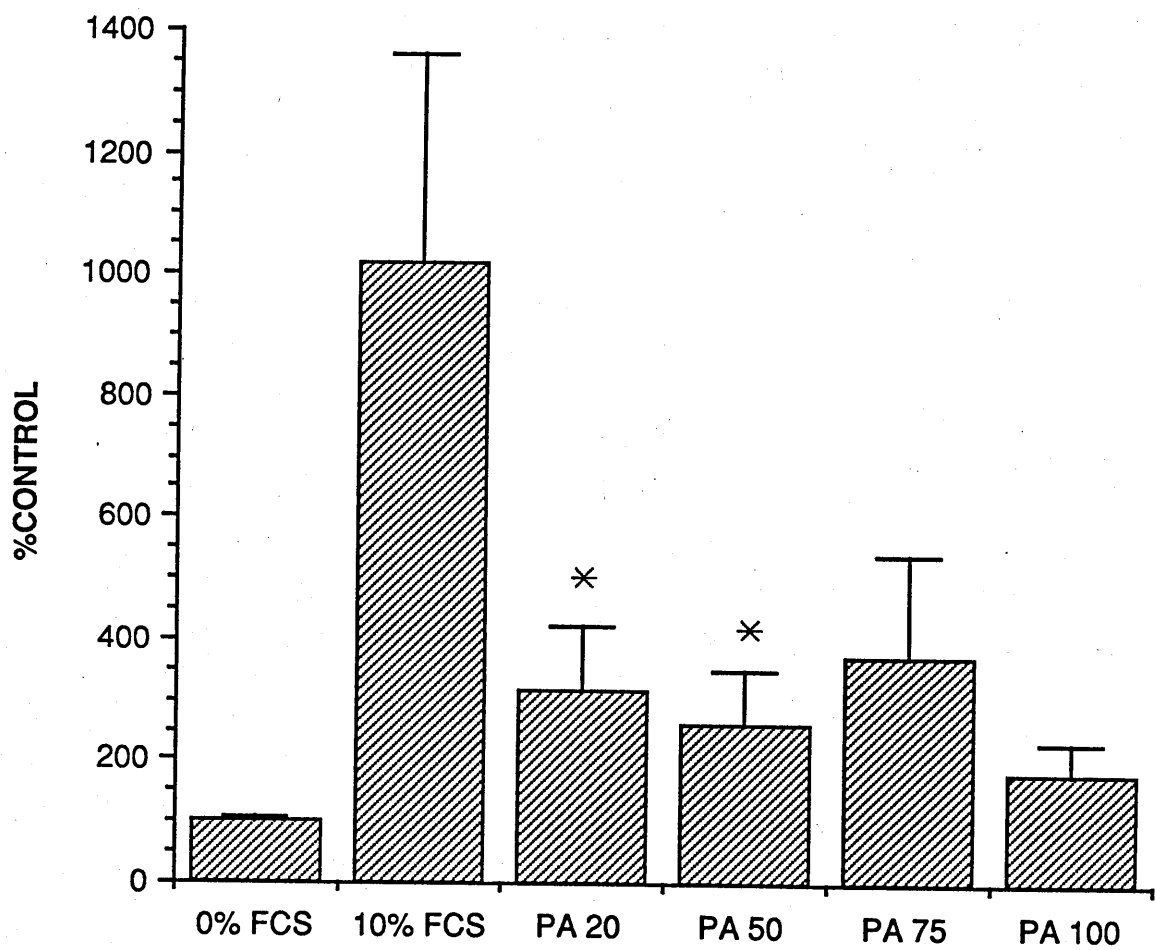
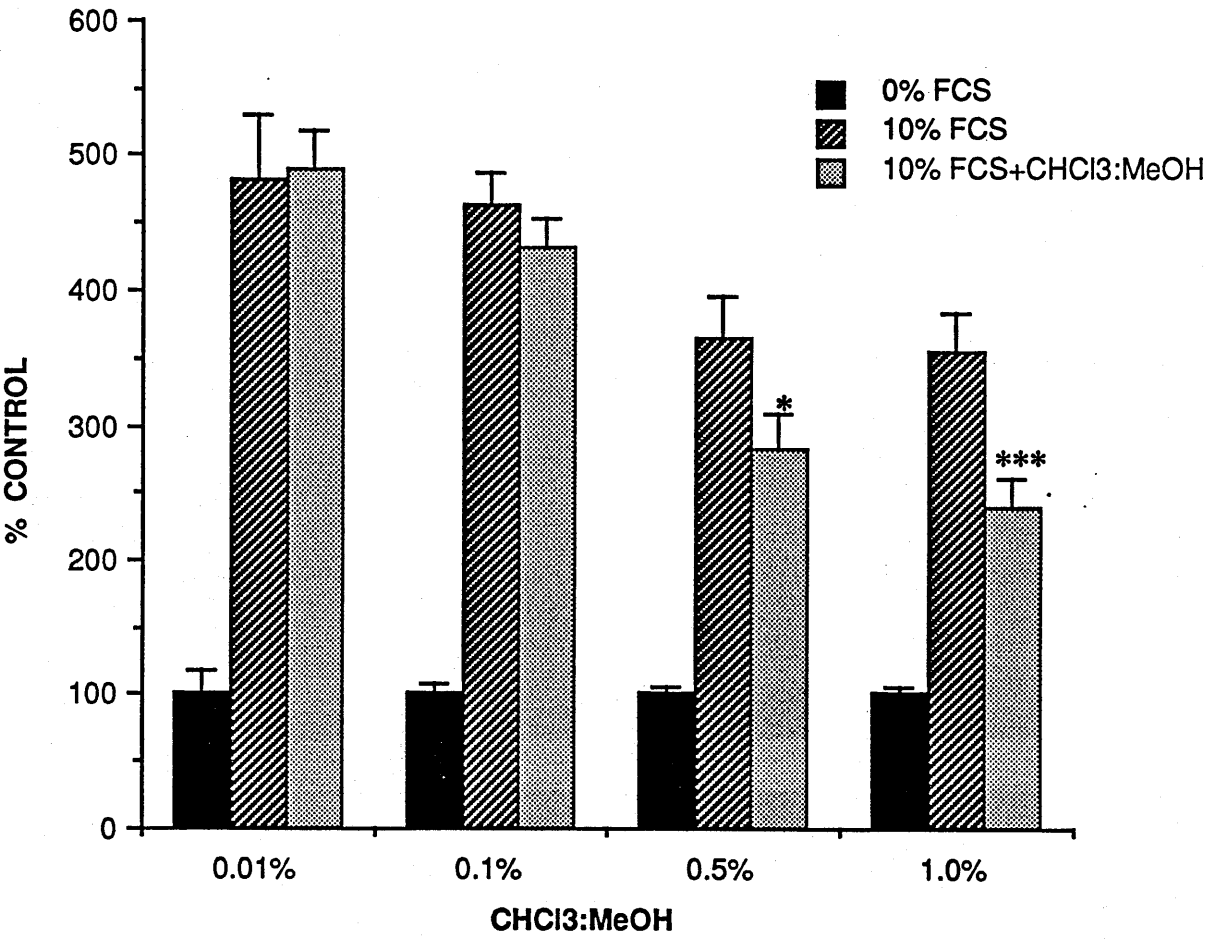


Fig. 2.8. The effect of increasing  $\text{CHCl}_3$ :MeOH (1:2 v/v) concentration on 10% FCS GM-induced  $[^3\text{H}]$ thymidine incorporation into astrocyte DNA.

Subconfluent astrocyte cultures (11 div) were serum depleted for 24h and then exposed to either 10% FCS GM alone or 10% FCS GM in the presence of increasing concentrations of  $\text{CHCl}_3$ :MeOH (1:2 v/v). Results are mean  $\pm$  SEM n = 8 from 1 experiment using 24 well plates and are expressed as % of GM-FCS control. GM-FCS mean dpm = 628 for 0.01%, 750 for 0.1%, 789 for 0.5% and 814 for 1.0%. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to appropriate 10% FCS GM control.

FIG. 2.8



the [ $^3\text{H}$ ]thymidine incorporation response to 10% FCS GM were seen in the presence of 0.5% and 1.0% v/v  $\text{CHCl}_3\text{:MeOH}$  (1:2 v/v) respectively (Fig. 2.9). 0.01, 0.1 and 0.5% v/v  $\text{CHCl}_3\text{:MeOH}$  (1:2 v/v) had no significant effect on the response of astrocytes to 10% FCS GM. Subsequent experiments were carried out using an aqueous suspension of PA.

The effect of PA obtained from Sigma in aqueous suspension on DNA synthesis of younger cultures of astrocytes, 5, 6 or 8 div was also investigated. PA at all concentrations tested was found to stimulate incorporation into cells grown for 5 div. 0.1 and  $1\ \mu\text{gml}^{-1}$  PA were found to increase [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA at 6 div but inhibit incorporation at 8 div. PA at 10 and  $100\ \mu\text{gml}^{-1}$  had no effect on 6 or 8 div cells (Fig. 2.10).

#### 2.3.3. The variation in the astrocyte proliferative response.

Variation was again seen in the response of astrocytes to 10% FCS GM with age in culture. Larger responses were seen to 10% FCS GM in this experiment with different age astrocyte cultures than in the previous experiment looking at the effect of PDGF on cultures of different ages. The [ $^3\text{H}$ ]thymidine incorporation experiments described above were performed using cells grown in 24 well plates. The data from the insulin experiment is shown in Table 2.3., it shows large variation in the dpm recovered from each of the 4 wells in each row that were treated with the same insulin

Fig. 2.9. The inhibitory effect of  $\text{CHCl}_3$ :MeOH (1:2 v/v) on the effect of 10% FCS GM on [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA.

Subconfluent astrocyte cultures (11 div) were serum depleted for 24h and then exposed to either 10% FCS GM alone or 10% FCS GM in the presence of increasing concentrations of  $\text{CHCl}_3$ :MeOH (1:2 v/v) for 48h. Results are mean  $\pm$  SEM n = 8 from 1 experiment using 24 well plates and are expressed as % of 10% FCS GM response. Mean dpm 10% FCS GM control = 3026 for 0.01%, 3479 for 0.1%, 2879 for 0.5% and 2887 for 1.0%. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to appropriate 10% FCS GM control.



FIG. 2.9

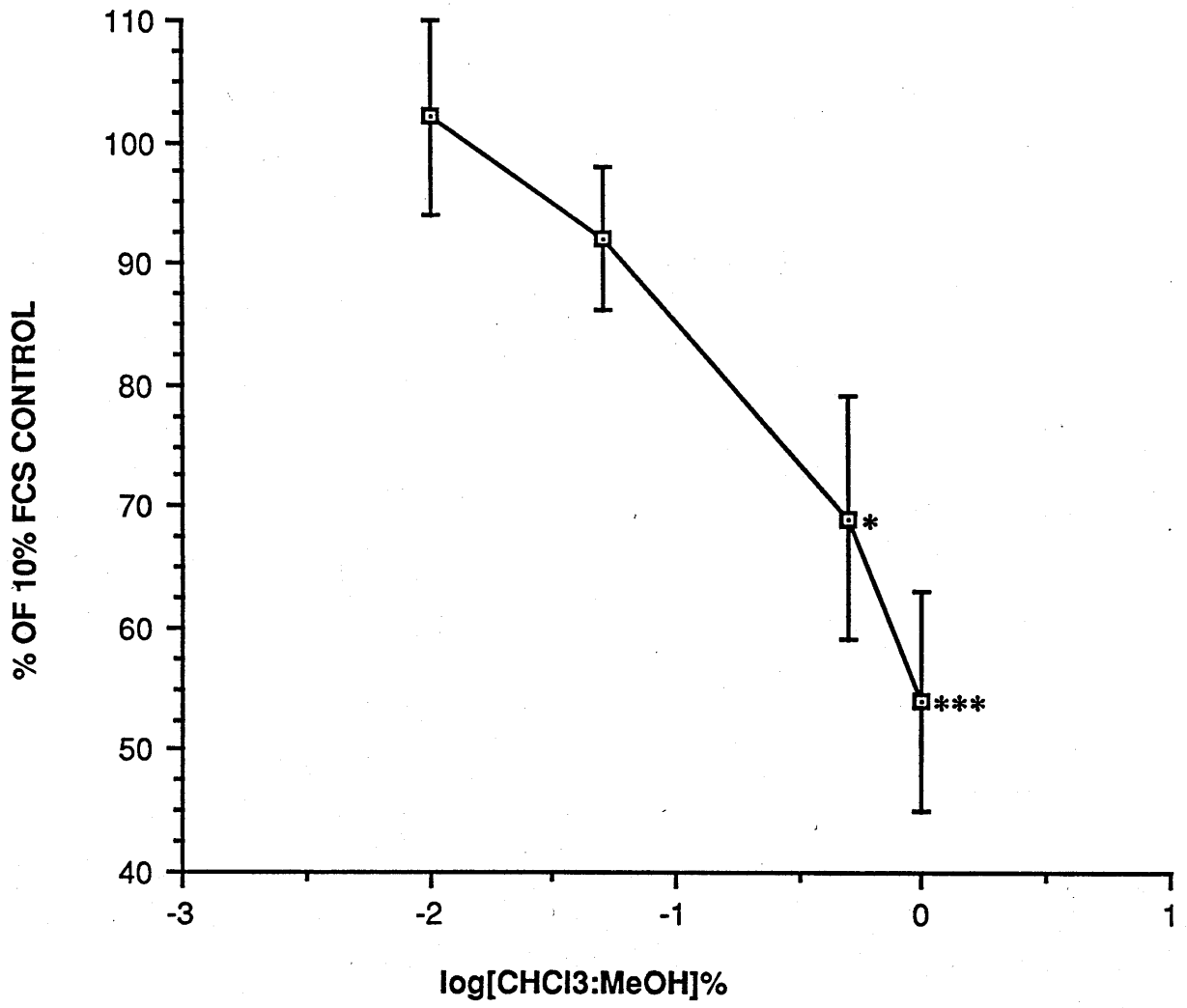


Fig. 2.10. PA-induced [<sup>3</sup>H]thymidine incorporation into 5, 6 and 8 div astrocytes.

Subconfluent astrocyte cultures (5, 6 or 8 div) were serum depleted and then exposed to PA at 0.1, 1, 10 or 100  $\mu\text{gml}^{-1}$  for 48h. Results are mean  $\pm$  SEM n = 3 from 1 experiment using 24 well plates and are expressed as % of GM-FCS control. GM-FCS mean dpm = 153 for 5 div, 232 for 6 div and 584 for 8 div. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to appropriate GM-FCS control.

FIG. 2.10

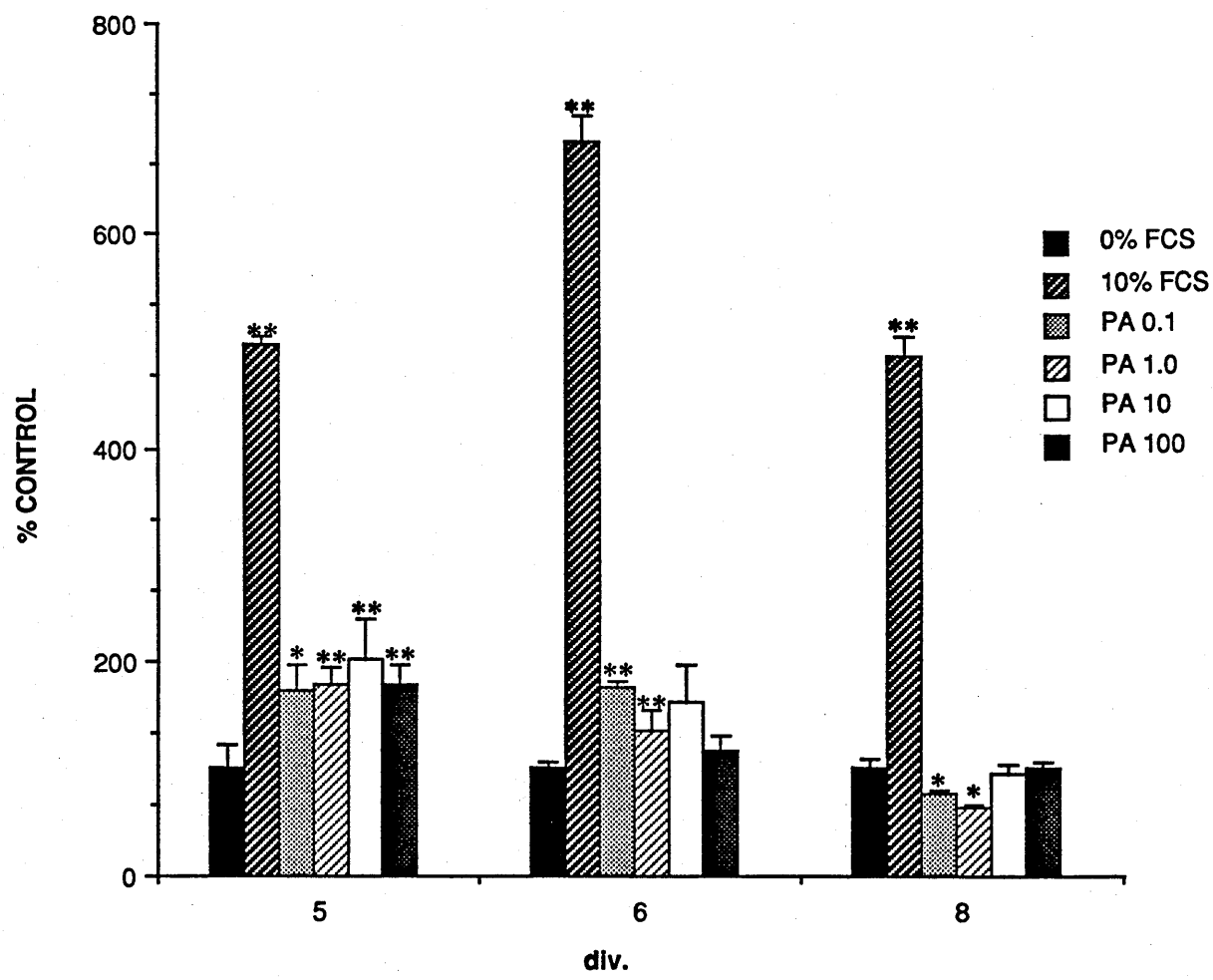


Table 2.3. Variation in dpm recovered from astrocyte cell cultures grown in 24 well plates.

Subconfluent astrocyte cultures (11 div) were serum depleted for 24h and then exposed to various concentrations of insulin for 48h. Results are dpm recovered from each well of two 24 well plates from 1 experiment.

	dpm							
	Plate 1				Plate 2			
GM-FCS	230	449	700	768	360	328	796	816
10% FCS GM	2169	2412	2314	2430	1901	2313	2602	2524
Insulin $\mu\text{Uml}^{-1}$								
1	635	942	806	838	752	1606	510	1178
10	901	1043	1546	1082	776	916	1318	1156
100	1163	2410	1885	1654	1274	2150	1895	1428
1000	1140	1872	2254	1789	1272	2374	1907	1707

concentration. For example the dpm recovered in response to the GM-FCS control varied from 230 to 768 dpm. The variation seen in the 10% FCS GM response of astrocytes could have been due to a large variation in the number of responsive cells in each well of these 24 well plates. The effect of 10% FCS GM on the incorporation of [<sup>3</sup>H]thymidine into cells in all 24 wells of two 24 well plates was therefore investigated.

As can be seen from Table 2.4. large variation in [<sup>3</sup>H]thymidine incorporation into DNA was found in response to 10% FCS GM across the 24 well plate. The incorporation varied from 669 to 3852 dpm on plate 1 and from 403 to 2451 dpm on plate 2. When the results from all 24 wells of each plate are combined the variation was equivalent to 10% of the mean value for plate 1 and 9% for plate 2, but if each row of 4 wells is looked at individually much greater variation is seen ranging from 13 to 32% of the mean. The [<sup>3</sup>H]thymidine incorporation experiments were carried out by making different additions to each row, but large variation in the number of responsive cells present in different rows of these 24 well plates were found. The 24 well plates seemed to be inherently variable so it was decided to look at astrocytes grown in 6 well (35mm diameter) plates to see if this problem could be overcome.

Table 2.4. Variation in 10% FCS GM-induced [<sup>3</sup>H]thymidine incorporation into astrocyte DNA.

Subconfluent astrocyte cultures (11 div) were serum depleted for 24h and then exposed to 10% FCS GM for 48h. Results are expressed as mean +/- SEM (n = 4) for each row of 24 well plate from 1 experiment. Variation = SEM as % of mean value.

Plate 1	dpm	Mean+/-SEM	Variation
10% FCS GM	1030 953 2197 1241	1355 +/- 287	21
	1965 1607 777 964	1328 +/- 277	21
	1770 3852 1623 823	2017 +/- 646	32
	1535 2312 1460 842	1537 +/- 301	20
	1617 1108 1373 509	1152 +/- 238	21
	669 1201 1262 1078	1053 +/- 133	13
	Overall	1407 +/- 143	10
Plate 2	dpm	Mean+/-SEM	Variation
10% FCS GM	573 1414 2451 1261	1425 +/- 388	27
	827 1021 1215 1370	1108 +/- 236	21
	987 1101 1517 766	1093 +/- 158	14
	794 1620 1147 590	1038 +/- 226	22
	1197 454 776 459	722 +/- 175	24
	403 724 801 600	632 +/- 87	14
	Overall	1003 +/- 95	9

#### 2.3.4. Comparison of astrocytes grown in 24 and 6 well plates.

The variability of subconfluent astrocyte cultures grown in 24 and 6 well plates was investigated by looking at the DNA content of each well of each plate. A typical standard curve for the standard calf thymus DNA preparation is shown in Fig. 2.11. The DNA content of an aliquot of the astrocyte cell suspension from each well was determined by comparison of its fluorescence with that of the calf thymus standard. From Table 2.5. it can be seen that there is much greater variation in the DNA content of the astrocyte cell suspension from a 24 well plate, the standard deviation (SD) being 85% of the mean compared to that from a 6 well plate, where the SD was only 13% of the mean. The DNA content of astrocyte cultures grown for different lengths time in 6 well plates was also determined (Fig. 2.12). From Fig. 2.13 it can be seen that the rate of proliferation changes with time in vitro. The assay of the DNA content of astrocyte cultures provided a less variable, simple and rapid method for the assessment of the growth factor-like action of PA. Consequently all further investigations were carried out using the convenient DNA content assay and the less variable astrocyte cultures grown in 6 well plates.

#### 2.3.5. Further investigations into the mitogenic action of PA on astrocytes.

The mitogenic action of PA (from Serdary Res. Labs) on astrocytes was assessed by measuring the DNA content after

Fig. 2.11. A typical standard curve for DNA from calf thymus.

Fluorescence of calf thymus DNA solution 0-5  $\mu\text{gml}^{-1}$  is shown. Fluorescence is expressed in arbitrary units.



FIG. 2.11

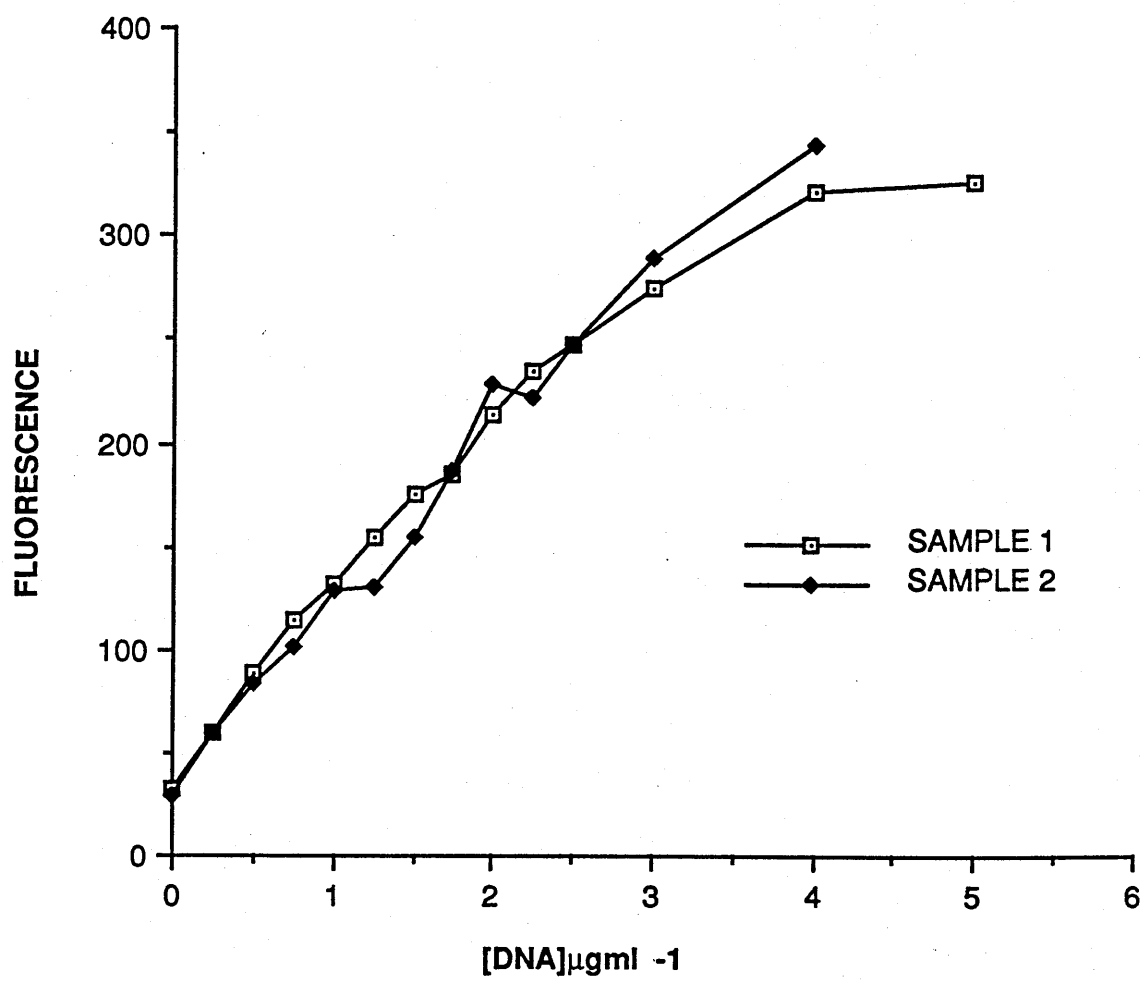


Table 2.5. DNA content of astrocyte cell suspension grown  
in 24 or 6 well plates.

The DNA content of an aliquot of each astrocyte cell  
suspension from each well was determined.

DNA  $\mu$ g/well

24 well (13 div)	0.14	0.38	0.00	0.21	0.06	0.00
	0.14	0.35	0.16	0.00	0.60	0.38
	0.42	0.40	0.06	0.31	0.54	0.16
	0.74	0.24	0.45	0.00	0.18	0.00

Mean +/- SD = 0.25 +/- 0.21 (100% +/- 85%) n = 24

6 well (13 div)	10.0	11.0	13.6
	13.6	12.8	14.0

Mean +/- SD = 12.5 +/- 1.63 (100% +/- 13%) n = 6

Fig. 2.12. DNA content of astrocyte cultures grown for different lengths of time in vitro.

The DNA content of astrocytes grown in vitro for varying lengths of time was determined. Results are expressed as mean  $\pm$  SEM (n = 6) from 1 experiment.

**FIG. 2.12**

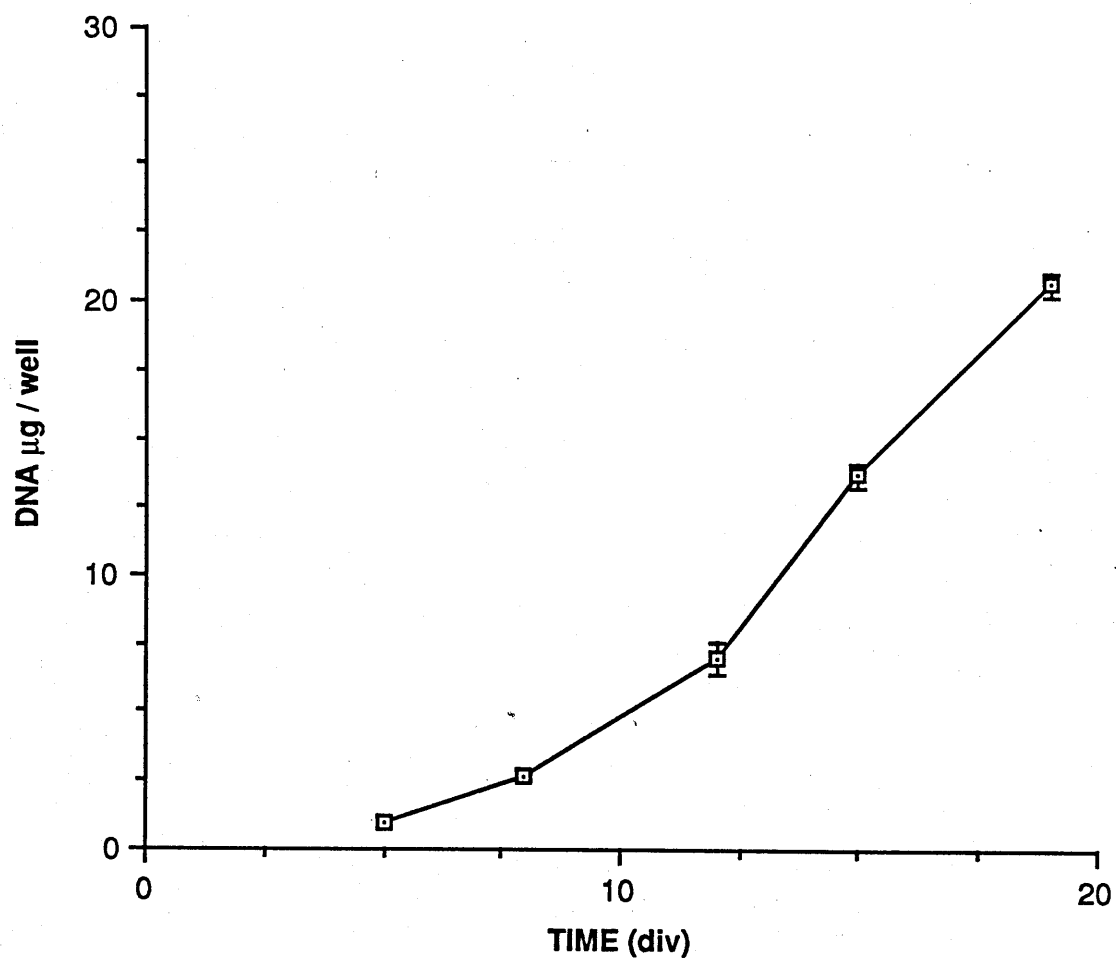
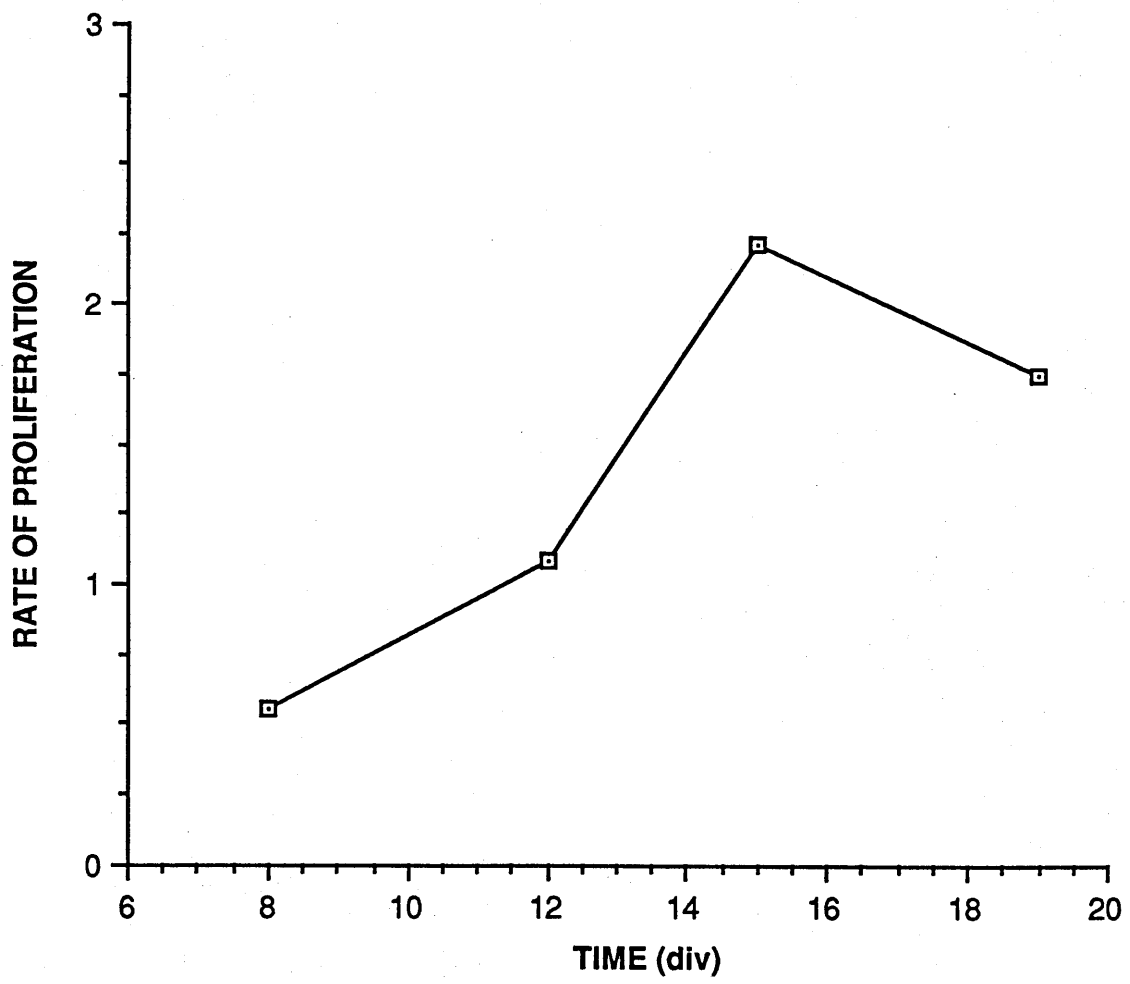


Fig. 2.13. The rate of proliferation of astrocytes in culture.

Results are mean  $\pm$  SEM  $n = 6$  and are expressed as the increase in DNA content divided by time in vitro.

**FIG. 2.13**



exposure of subconfluent astrocyte cultures (11 div) to PA for 48h. The fluorescence of the 10% FCS GM, 0.1% FCS GM and  $100\mu\text{gml}^{-1}$  PA exposure medium was also examined. As can be seen from Table 2.6. 0.1% FCS GM, 10% FCS GM and PA at  $100\mu\text{gml}^{-1}$  all fluoresce and consequently could interfere with the determination of the DNA content of the astrocyte cell suspension. Thorough washing of the astrocyte cultures with PSS after exposure and prior to the determination of the DNA content, to remove all traces of the exposure medium was therefore very important. The fluorescence of these washes was also checked and as can be seen from Table 2.6. this was negligible, indicating that the measured fluorescence of the astrocyte cell suspension was an accurate measure of DNA content and was not due to contamination of the astrocyte cell suspension with exposure medium.

Exposure of astrocyte cultures, that had been serum depleted for 24h, to PA at  $100\mu\text{gml}^{-1}$  for 48h elicited an increase in DNA content of 13% over the 0.1% FCS GM control (Fig. 2.14). A 51% increase in DNA content was found in response to 10% FCS GM. The response to PA was equivalent to 25% of the response to 10% FCS GM. PA at  $10\mu\text{gml}^{-1}$  caused a small but significant increase in DNA content of 11 div cells after 48h exposure (Fig. 2.15) but PA at  $1\mu\text{gml}^{-1}$  had no effect.

In an attempt to amplify PA's mitogenic activity the effect of increasing the time of serum depletion was investigated. For these experiments the cells were serum depleted in GM containing 0.1% FCS rather than 0% FCS because of the

Table 2.6. Fluorescence of exposure medium and PSS washes of astrocyte cultures.

An aliquot of the exposure medium and the subsequent PSS washes was taken and the fluorescence determined in duplicate and the equivalent DNA content calculated by comparison with the DNA standard.

Exposure medium	Equivalent DNA content ( $\mu\text{gml}^{-1}$ )
0.1% FCS GM	1.55
10% FCS GM	2.35
PA 100 $\mu\text{gml}^{-1}$	0.65
PSS	0.00
Washes from 0.1% FCS GM	0.00
10% FCS GM	0.00
PA 100 $\mu\text{gml}^{-1}$	<0.10



Fig. 2.14. DNA content of astrocyte cultures depleted of serum for varying lengths of time.

Subconfluent cultures were serum depleted for varying lengths of time and then exposed to PA at  $100\mu\text{gml}^{-1}$  for 48h. Results are expressed as mean  $\pm$  SEM.  $n = 4$  wells for each condition, except for 48h, 11 div  $n = 6$  wells.

0.1% FCS GM control 24h, 11 div  $3.31\mu\text{g DNA/well}$

48h, 11 div 4.40

72h, 12 div 9.33

Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to appropriate 0.1% FCS GM control.

**FIG. 2.14**

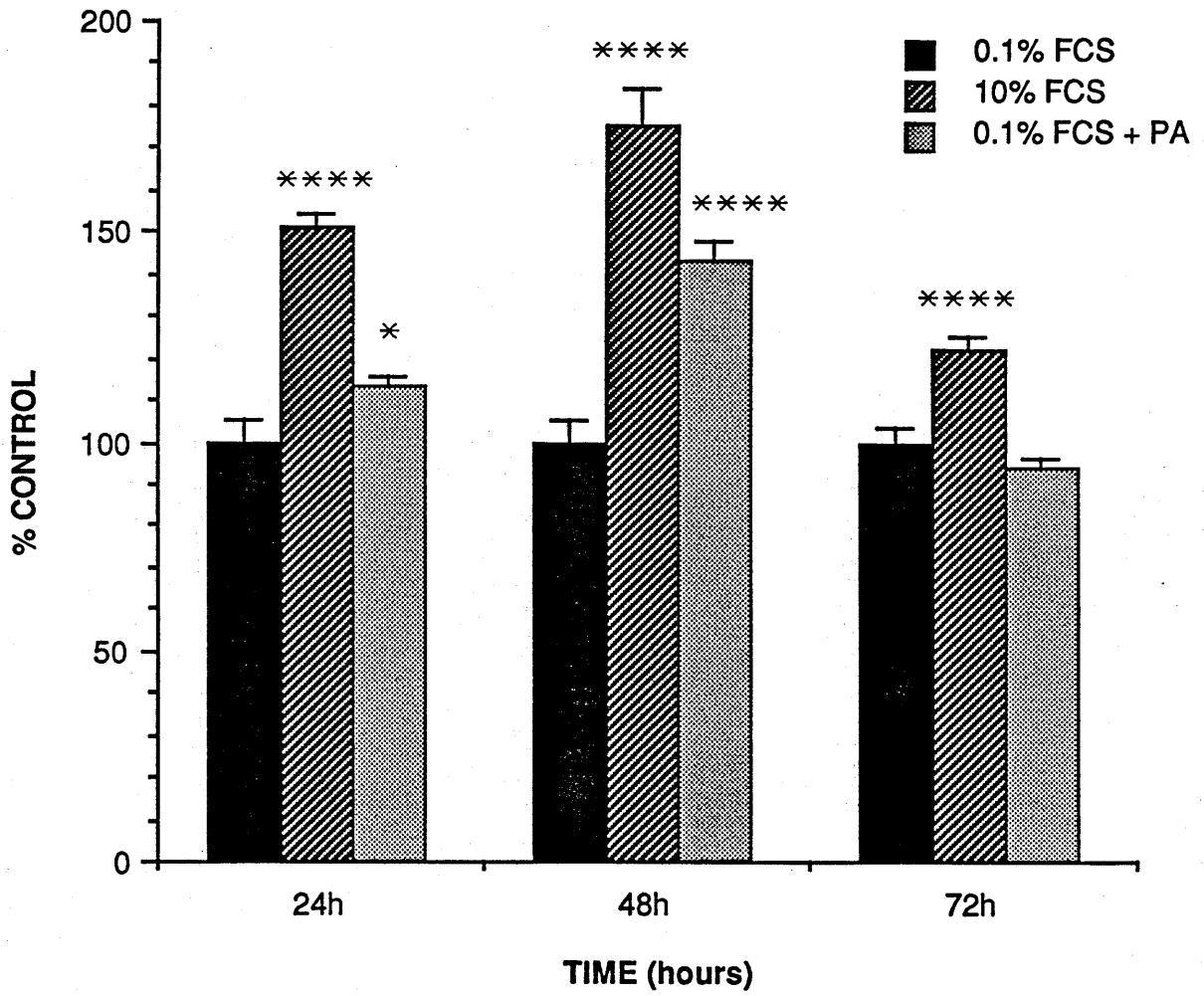
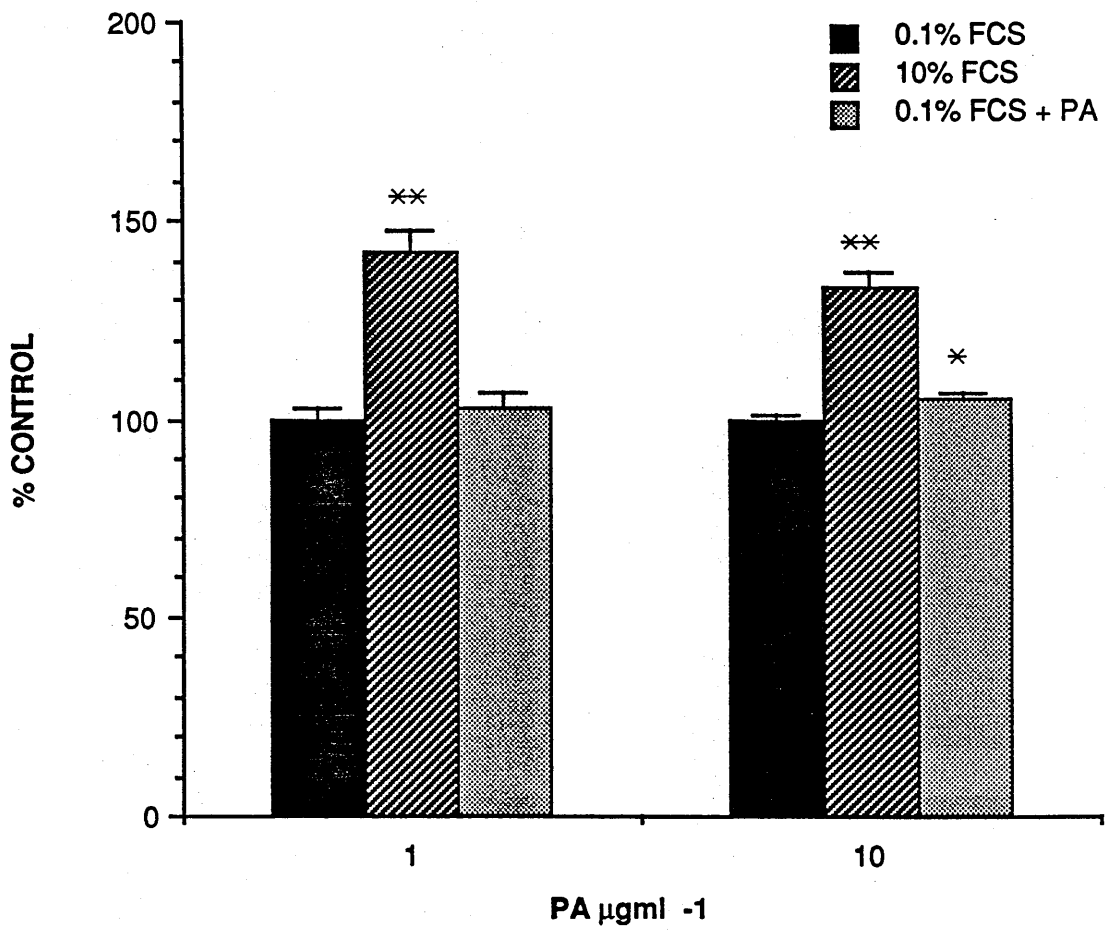


Fig. 2.15. DNA content of astrocytes exposed to PA at 1 and  $10\mu\text{gml}^{-1}$ .

Subconfluent cultures (11 div) were serum depleted for 48h and then exposed to PA at 1 or  $10\mu\text{gml}^{-1}$  for 48h. Results are mean  $\pm$  SEM (n = 4 wells) and are expressed as % of 0.1% FCS GM. 0.1% FCS GM control  $7.58\mu\text{g}$  DNA/well for PA 1,  $6.30\mu\text{g}$  DNA/well for PA 10. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to appropriate 0.1% FCS GM control.

**FIG. 2.15**



increased time of depletion. Increasing the time of depletion from 24h to 48h increased the response elicited by PA at  $100\mu\text{gml}^{-1}$  from a 13% increase to a 43% increase over the 0.1% FCS GM control (Fig. 2.14). An increase in the response to 10% FCS GM was also seen from 51% to 75% over the 0.1% FCS GM control. After 48h serum depletion the response to PA was equivalent to 57% of that elicited by 10% FCS GM. Increasing the time of serum depletion still further to 72h however had a detrimental effect, eliminating the mitogenic activity of PA and reducing the 10% FCS GM response to less than that seen after only 24h depletion.

The growth factor-like action of PA of differing fatty acid chain length composition was determined by exposing cells to PA at  $100\mu\text{gml}^{-1}$  containing different fatty acids for 48h (Table 2.7.). Only PAs containing longer chain fatty acids, that is PA from lecithin and PA containing oleic acid ( $\text{C}_{18:1}$ ) caused increases in the DNA content of astrocyte cultures. PA containing palmitic ( $\text{C}_{16:0}$ ), myristic ( $\text{C}_{14:0}$ ) or lauric ( $\text{C}_{12:0}$ ) acid had no effect on DNA content.

2.3.6. The effect of phorbol ester pretreatment on the mitogenic action of PA on astrocytes.

The contribution made by PKC activation to the mitogenic action of PA on astrocytes was assessed by treating the astrocyte cultures with the phorbol ester PMA prior to PA addition. This was achieved by incubating cultures with  $1\mu\text{M}$  PMA in 0.1% FCS GM for 6h, and then in fresh 0.1% FCS GM for 42h, prior to exposure to PA at  $100\mu\text{gml}^{-1}$  for 48h. The

Table 2.7. DNA content of astrocytes exposed to different fatty acid chain length PAs.

Subconfluent astrocyte cultures (11 div) were serum depleted for 48h and then exposed to PAs of differing composition for 48h. Results are mean  $\pm$  SEM n = 4 wells and are expressed as DNA  $\mu$ g/well and as % of 0.1% FCS GM control. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to the 0.1% FCS GM control.

Sample	DNA $\mu$ g/well	% of 0.1% control
0.1% FCS GM	13.70 $\pm$ 0.25	100 $\pm$ 2
10% FCS GM	21.14 $\pm$ 0.43	154 $\pm$ 3
PA C <sub>12:0</sub>	13.40 $\pm$ 0.70	98 $\pm$ 5
PA C <sub>14:0</sub>	14.30 $\pm$ 1.10	104 $\pm$ 8
PA C <sub>16:0</sub>	14.80 $\pm$ 0.80	108 $\pm$ 6
PA C <sub>18:1</sub>	15.50 $\pm$ 0.40	113 $\pm$ 3 *
PA lecithin	15.30 $\pm$ 0.80	112 $\pm$ 6 *

response of phorbol ester-treated astrocytes to PA was compared to that of untreated cells. PA elicited a 20% increase over the 0.1% FCS GM control in the DNA content of untreated astrocyte cultures (Fig. 2.16). PMA pretreatment abolished the mitogenic action of PA on astrocytes. In untreated cells 10% FCS GM elicited a 66% increase in DNA content over the 0.1% FCS GM control, whereas, in treated cells 10% FCS GM elicited only a 39% increase over the control.

Fig. 2.16. DNA content of astrocytes exposed to  
PA +/- PMA pretreatment.

Subconfluent cultures (11 div) were either serum depleted only for 48h or treated with PMA ( $1\mu\text{M}$  in 0.1% FCS GM) for 6h and then serum depleted for 42h. Treated and untreated cells were then exposed to PA at  $100\mu\text{gml}^{-1}$  for 48h. Results are mean +/- SEM n = 8 wells from 2 experiments and are expressed as % of 0.1% FCS GM control.

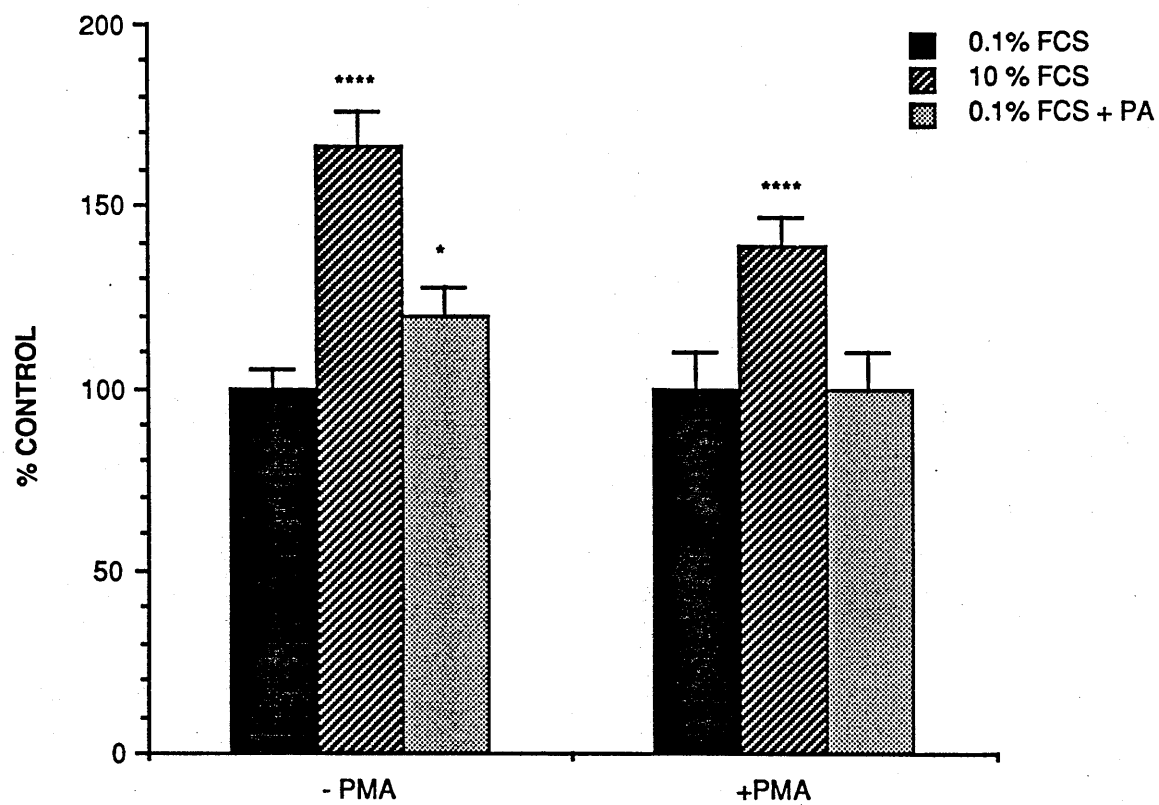
0.1% FCS GM control - PMA  $4.09\mu\text{g}$  DNA/well

+ PMA  $4.33\mu\text{g}$  DNA/well

Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to the appropriate 0.1% FCS GM control.



FIG. 2.16



## 2.4. SUMMARY

(i) Insulin elicited a dose-dependent increase in [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA. PDGF and PA both had a biphasic effect on [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA due to the presence of high concentrations of their respective vehicles, ethanediol and  $\text{CHCl}_3\text{:MeOH}$  (1:2 v/v) at the higher PDGF and PA concentrations tested.

(ii) PDGF had an inhibitory effect on [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA of cells grown for 5 div whereas PA was stimulatory at this time. PDGF had no effect on those cells grown for 6 or 8 div and the lower concentrations of PA that were tested had a stimulatory effect at 6 div and an inhibitory effect at 8 div.

(iii) Large variation was seen in the 10% FCS-GM stimulated [ $^3\text{H}$ ]thymidine incorporation into astrocytes grown in 24 well plates. This prompted the use of the less variable 6 well plates and the simple and rapid DNA assay method.

(iv) PA was shown to be mitogenic using this DNA content assay and that the signal could be amplified by increasing the time of serum depletion from 24 to 48h. This effect of PA was dependent on the presence of PA containing long chain fatty acids.

(v) Phorbol ester pretreatment abolished the mitogenic action of PA on astrocytes and reduced that of 10% FCS-GM.

## 2.5. DISCUSSION

The presence of structurally, physiologically and biochemically distinct high affinity receptors for insulin in glial (Clarke et al 1984; 1988) and neuronal cell cultures (Boyd Jr. et al 1985) has raised questions about the physiological role of insulin in the CNS. The presence of insulin-like immunoreactivity in primary cultures of neurons from rat brain (Raizada 1983) and the release of immunoreactive insulin from neuronal cells in culture after depolarisation could provide a means of communication between neuronal and glial cells (Clarke et al 1986).

This communication between neuronal and glial cells could be involved in the control of the growth and development of the CNS. Insulin, at physiological concentrations, was found to be a potent glial mitogen in this study as well as in a number of others (Morrison and de Vellis 1981; Lenoir and Honegger 1983; Morrison et al 1985; Murphy et al 1987; Shemer et al 1987) and has been found to enhance astroglial proliferation and survival in culture (Sensenbrenner et al 1986), although it was reported to have no mitogenic action by some other workers (Pruss et al 1982; Avola et al 1988; Kniss and Burry 1988). Insulin also had a proliferative action on primary cultures of 1 day old neonatal rat neurons (Shemer et al 1987) and the survival of 19 day foetal neurons required the addition of insulin (Kadle et al 1988). Neonatal neuronal cultures also proliferate in response to IGF I (Shemer et al 1987). IGF I and IGF II also act as glial mitogens (Lenoir and Honegger 1983). Insulin and IGF

I are also thought to play a role in oligodendrocyte development (Van der Pal et al 1988). When used at physiological concentrations, that is, of between 2 and 6nM insulin binds to insulin receptors only but at higher concentrations it can also bind to the IGF I receptor (Clarke et al 1984). Insulin may be having its physiological action by binding to its own receptor where it is added at physiological concentration (Murphy et al 1987) but at higher concentrations (Morrison and de Vellis 1981; Lenoir and Honegger 1983; Morrison et al 1985; Shemer et al 1987) it may be acting by binding to the IGF I receptor. In the studies by Pruss et al (1982), Avola et al (1988) and Kniss and Burry (1988), where a lack of proliferative action of insulin was seen, insulin degradation could not have been the cause since it was present at concentrations above those thought physiological and half of the immunoreactive insulin added has been found to still be present in the culture after 24h (Lenoir and Honegger 1983).

The mechanism of growth factor action of insulin on astrocytes is unknown, but in other cell types it has been found to involve protein tyrosine kinase activation (Kasuga et al 1982). There has been some controversy over whether insulin is capable of activating PKC (Spach et al 1986; Cooper et al 1987) and PPI turnover (Pennington and Martin 1985; Farese et al 1986). It is now thought that the actions of insulin are brought about by the hydrolysis of glycosyl-phosphatidylinositol by a specific PL C to generate an inositol phosphate glycan and a structurally distinct DG (Saltiel and Cuatrecasas 1988). This inositol phosphate

glycan mimicks certain actions of insulin and the distinct DG may be involved in the activation of one or more of the PKCs that have been identified (Nishizuka 1988).

Further work is obviously required to elucidate the physiological significance of insulin in the CNS in vivo but its release from neurons and its proliferative action on astrocytes does suggest a putative role in the control of astroglial growth.

PDGF receptors have been found on a number of different astroglial preparations (Herschmann 1986) and PDGF has been reported to act as a glial mitogen (Besnard et al 1987; Noble et al 1988; Yong et al 1988) as was found in this study. The PDGF preparation used in this study however elicited a biphasic dose response curve. At higher PDGF concentrations a lower than expected astroglial proliferative response was seen. It appears from preliminary studies that this may have been due to the presence of ethanediol which was used as preservative in the PDGF preparation. At the higher PDGF concentrations that were tested ethanediol was present at a concentration that was found to reduce the effect of 10% FCS GM on astroglial proliferation.

The response to PDGF in this study seemed to vary with astrocyte age in culture but the pattern observed was in general agreement with previously reported results (Besnard et al 1987). PDGF had no effect on cells grown for 6 or 8 div but did stimulate incorporation into cells grown for 11

div. Besnard et al (1987) found that astrocytes were unresponsive at 5 and 15 div but maximally stimulated at 10 div. PDGF has been found to influence glial development in the rat optic nerve (Noble et al 1988). Type 1 astrocytes secrete a soluble factor, which has been identified as PDGF, which is capable of promoting the division and motility of oligodendrocyte-Type 2 astrocyte (O-2A) progenitor cells, and of inhibiting the premature differentiation of O-2A cells into oligodendrocytes. Antibodies to PDGF have been found to block the action of Type 1 astrocytes on O-2A cells (Noble et al 1988). There is evidence from murine teratocarcinoma stem cells, which are used as a model to study the development of the mouse blastocyst, that a transition from a PDGF-producing to a PDGF-responsive cell type may be possible (Ross et al 1986). PDGF produced by Type 1 astrocytes may be involved in gliogenesis during development and later they may become responsive to PDGF as part of their role in the response to CNS injury thus explaining the age dependent effects of PDGF on astroglial proliferation.

A role for PDGF in the reactive gliosis that is seen in response to CNS injury has been proposed by Besnard et al (1987). PDGF released by platelets at the site of injury penetrates into the surrounding brain tissue through edema fluid (Takamiya et al 1986) may, by acting as a mitogen and chemoattractant, stimulating adjacent cells to divide and form the characteristic glial scar.

The mitogenic action of PA was established using

[<sup>3</sup>H]thymidine incorporation and DNA content of astrocyte cultures. Murphy and Pearce (1988a) showed a dose-dependent increase in incorporation into astrocyte cultures but in this study the results from the incorporation study were complicated by the presence of CHCl<sub>3</sub>:MeOH at the higher PA concentrations tested. An inhibitory effect of CHCl<sub>3</sub>:MeOH on FCS-induced proliferation was seen and ethanol has also been reported to inhibit astroglial proliferation (Davies and Vernadakis 1984). However, a dose-dependent increase in DNA content of astrocyte cultures in response to aqueous PA was seen. Differences were also seen in the response of cells of different ages to PA. Unlike the inhibitory effect that PDGF had on 5 div astrocytes these cells responded to all PA concentrations tested. Differences were also seen in the response of 6 and 8 div cells to PA from those to PDGF. At 6 div a stimulatory effect was seen at lower PA concentrations but these concentrations were inhibitory at 8 div, whereas PDGF had no effect on these cells.

This mitogenic action of PA on astrocytes appears to be dependent on the presence of long chain fatty acids. Only PA containing C<sub>18:1</sub> and PA produced from lecithin increased DNA content, whereas PA containing C<sub>12:0</sub>, C<sub>14:0</sub> or C<sub>16:0</sub> fatty acids were ineffective. This requirement for longer chain length fatty acids has also been seen in other cell types. In NIH 3T3 cells PA containing C<sub>12:0</sub> or C<sub>14:0</sub> fatty acids had little effect on [<sup>3</sup>H]thymidine labelling (Yu et al 1988). This effect also seems to be specific for the phospholipid PA. Yu et al (1988) found that phosphatidylcholine (PC), phosphatidylethanolamine (PE) and

lysoPE had little effect on NIH 3T3 cells and [<sup>3</sup>H]thymidine incorporation into Rat-1 fibroblasts was not effected by phosphatidylinositol (PtdIns) or phosphatidylserine (PS) (Moolenaar et al 1986). Liposomes made of PA and PC but not those of PS and PC were able to stimulate [<sup>3</sup>H]thymidine incorporation into Swiss 3T3 cells. PA and PC were also presented to these cells in aqueous buffer rather than in liposomes and still stimulated DNA synthesis (Siegmann 1987). The ganglioside GM1 has been shown to stimulate astroglial proliferation (Skaper and Varon 1987). GM1 and PA share some structural similarity in that they both possess lipophilic and hydrophilic domains. PA in common with GM1 is able to inhibit the cAMP-dependent conversion of flat, epithelial-like astroglia to process-bearing, stellate astroglia. This PA effect is again dependent on the presence of longer chain length fatty acids and appears to be specific for PA since PC, PE, PtdIns and PS were without effect (Skaper et al 1989). Endogenously produced PA, formed by the action of phospholipase D (PL D) on membrane phospholipids, was also able to inhibit this cAMP-dependent morphological conversion. All these reports support the idea that the stimulation is specific for PA.

Serum is capable of stimulating PPI turnover in fibroblasts (L'Allemain and Pouyssegur 1986; Moolenaar et al 1986) and consequently PPI hydrolysis may be permanently switched on in cells in the presence of serum. Serum contains phospholipids (Krause and Debuch 1987) and by increasing the period of serum depletion progressive enhancement of the effect of agents known to stimulate PPI turnover may be



seen. Thrombin stimulates PPI turnover in fibroblasts and increasing growth factor deprivation results in a progressive increase in the effect of thrombin on PPI hydrolysis (L'Allemain et al 1986). Thrombin also has a proliferative effect on astroglial cells (Perraud et al 1987). PA is proposed to elicit its growth factor-like action by stimulating PPI turnover. Could the effect of PA be amplified like that of thrombin? By varying the time of serum depletion prior to PA exposure an amplification of the response to PA was seen. The growth medium itself which was used for culturing astrocytes was found to contain PA. 23% of the radioactivity recovered in the phospholipid fraction of growth medium labelled with [ $^3\text{H}$ ]arachidonic acid ([ $^3\text{H}$ ]AA) was associated with PA (see later, chapter 4). Desensitisation to PA might therefore have occurred which was alleviated by increasing the time of serum depletion.

PKC activation has been demonstrated to play a role in the proliferative response of a number of cell types (Nishizuka 1986). Activation of PKC by DG is thought to activate the  $\text{Na}^+/\text{H}^+$  antiporter (Moolenaar 1986) which is involved in cytosolic alkalinisation (Moolenaar et al 1983) required for the initiation of DNA synthesis. Phorbol ester, a direct activator of PKC in vivo and in vitro (Castagna et al 1982), has been found to mimick the action of growth factors in raising  $\text{pH}_i$  (Moolenaar et al 1984a). The presence of PKC in primary cultures of astrocytes (Neary et al 1986; Murphy et al 1988) and phorbol ester's ability to stimulate proliferation of glial cells (Murphy et al 1987; Bhat 1989) suggest a role for PKC activation in the control of glial

cell growth. PKC activation is also implicated in astroglial growth from the observation that FCS increases the activity of the  $\text{Na}^+/\text{H}^+$  antiporter (Skaper and Varon 1987). Further evidence is the ability of 1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine, H7, a potent PKC inhibitor (Halenda and Rehm 1987) to block the effect of PMA on glial cell proliferation (Bhat 1989).

Prior incubation with PMA abolished PA's proliferative action on astrocytes. In astrocytoma and astroglial cells the early phase of PKC activation by phorbol ester produces a positive response which is then followed by negative feedback control of some cell surface receptors (Orellana et al 1985; Pearce et al 1988). This down-regulation has been found to be a major function of PKC activation (Nishizuka 1986). PA has been shown to cause PKC-mediated activation of  $\text{Na}^+/\text{H}^+$  exchange in A431 human carcinoma cells and the mechanism of action of PA has been proposed to be via stimulation of PPI turnover (Moolenaar et al 1986). The abolition of PA's mitogenic action on phorbol ester pretreated astroglial cells could be attributed to the down-regulation of PA cell surface receptors. This suggests that PKC activation does appear to be involved in the growth factor action of PA on astrocytes in culture. The phorbol ester PMA has been found to have a mitogenic effect on astrocytes (Murphy et al 1987; Bhat 1989) although at concentrations lower than that employed for phorbol ester pretreatment studies. However, PMA pretreatment of Swiss 3T3 cells had no effect on PA's ability to stimulate proliferation in these cells (Yu et al 1988). There may be

cell specific differences in the mechanisms used by PA to elicit its growth factor action. Some 3T3 cells may not depend upon stimulation of phospholipid metabolism for their response to mitogenic signals (Yu et al 1988). Growth factor regulation of PKC may also differ in cells that are still capable of proliferating and those that are differentiated (Morrison et al 1988). Phorbol ester is long-lived in the cell when compared to the rapidly produced and degraded DG. Consequently studies using phorbol esters may have different effects to that of DG and this reservation must be expressed in connection with these studies. PKC activation and  $\text{Ca}^{++}$  mobilisation act in synergy to bring about the cellular response but the initial increase in  $[\text{Ca}^{++}]$  is transient so PKC activation and the subsequent phosphorylation of target proteins may be required to ensure that the long term responses such as proliferation occur.

Astrocytes have been shown to proliferate in response to a number of agents that are thought to act via the activation of PPI turnover, for example PDGF, serum and thrombin. The activation of PPI turnover does not however automatically mean that a particular agent will also elicit astroglial proliferation. Bradykinin and vasopressin have been shown to stimulate PPI turnover in astrocytes but have no effect on astroglial proliferation (Cholewinski and Wilkin 1988). Carbachol, histamine, noradrenaline and serotonin, agents known to activate inositol phospholipid hydrolysis in astrocytes (Pearce et al 1985; 1986a) have also been shown to have no effect on astroglial proliferation (Murphy et al

unpubl.). However, recent results show that carbachol and noradrenaline are capable of stimulating DNA synthesis in primary astrocytes derived from neonatal rat brain (Ashkenazi et al 1989). The action of carbachol was mediated by  $M_1$  and  $M_4$  muscarinic acetylcholine receptor subtypes. These receptor subtypes were shown to be more efficient at activating PPI hydrolysis than the  $M_2$  or  $M_3$  receptor subtypes. The degree to which carbachol stimulated DNA synthesis was well correlated with the degree of PPI hydrolysis activation. Carbachol was also mitogenic for SK-N-SH human neuroblastoma cells and 1321 N1 human astrocytoma cells which possess  $M_4$  muscarinic receptors. The reason for the inability of carbachol to stimulate DNA synthesis in the study of Murphy et al is unknown since these cells do express  $M_1$  receptor subtypes and carbachol-induced PPI turnover was found to be mediated by the  $M_1$  receptor subtype (Murphy et al 1986). Serotonin, although having no effect on astroglial proliferation, has been reported to be a mitogen for Chinese hamster lung fibroblasts (Seuwen et al 1988). Serotonin is thought to act by stimulating PPI turnover because no synergy was seen with thrombin, suggesting that they share a common pathway of signal transduction and because serotonin was able to potentiate the action of EGF, FGF and insulin which act via protein tyrosine kinase stimulation.

As mentioned above PA is thought to elicit its growth factor-like action via the stimulation of PPI turnover (Moolenaar et al 1986). A number of agents that activate PPI hydrolysis, for example, carbachol, noradrenaline, PDGF,

serum and thrombin do have a mitogenic action on astrocytes (Besnard et al 1987; Murphy et al 1987; Perraud et al 1987; Ashkenazi et al 1989). Does PA which is also mitogenic for astrocytes (Murphy and Pearce 1988a; this study) also activate inositol phospholipid hydrolysis in astrocytes? Investigations into whether PA is capable of stimulating PPI turnover in astrocytes are described in the following chapter.

## CHAPTER 3. PHOSPHATIDIC ACID STIMULATES PHOSPHOINOSITIDE TURNOVER AND $\text{Ca}^{++}$ MOBILISATION IN ASTROCYTES.

### 3.1. INTRODUCTION

In the previous chapter the mitogenic action of PA on astrocytes, initially reported by Murphy and Pearce (1988a), was confirmed with the finding that PA increased [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA and increased the DNA content of astrocyte cultures. The abolition of the mitogenic action of PA by PKC down-regulation suggests a role for PPI turnover in the effect of PA on astroglial proliferation.

The mechanism of action of a number of mitogenic agents, some of which have been shown to be astrocyte mitogens, is thought to involve the activation of PPI turnover. A number of other mitogens are thought to employ the activation of protein tyrosine kinase (see Chapter 2 Table 2.2.). The ability of a number of mitogens to stimulate proliferation has been well correlated with their action on PPI turnover (Whitman and Cantley 1988; Ashkenazi et al 1989; Chapter 2 Table 2.2.). PDGF and thrombin, which are astrocyte and fibroblast mitogens (Scher et al 1979; Perez-Rodriguez et al 1981; Ek et al 1982; Besnard et al 1987; Perraud et al 1987; Noble et al 1988; Yong et al 1988), have been found to activate PPI breakdown in fibroblasts (Habenicht et al 1981; Berridge et al 1984; Hesketh et al 1988) and in platelets and fibroblasts respectively (Billah and Lapetina 1982; Agranoff et al 1983; L'Allemain et al 1986). Serum, which

is a potent astrocyte mitogen (Murphy et al 1987; Skaper and Varon 1987; Avola et al 1988; Kniss and Burry 1988), is also capable of stimulating PPI breakdown (Moolenaar et al 1985 and 1986; L'Allemain et al 1986). Other mitogens, for example, bradykinin, vasopressin and bombesin (Rozengurt et al 1979; Owen and Villereal 1983; Rozengurt and Sinnett-Smith 1983) are also thought to use the activation of PPI turnover to bring about their proliferative action (Berridge and Irvine 1984; Yano et al 1984; Hesketh et al 1988). It has been proposed that the mitogenic action of PA involves the stimulation of PPI turnover (Moolenaar et al 1986).

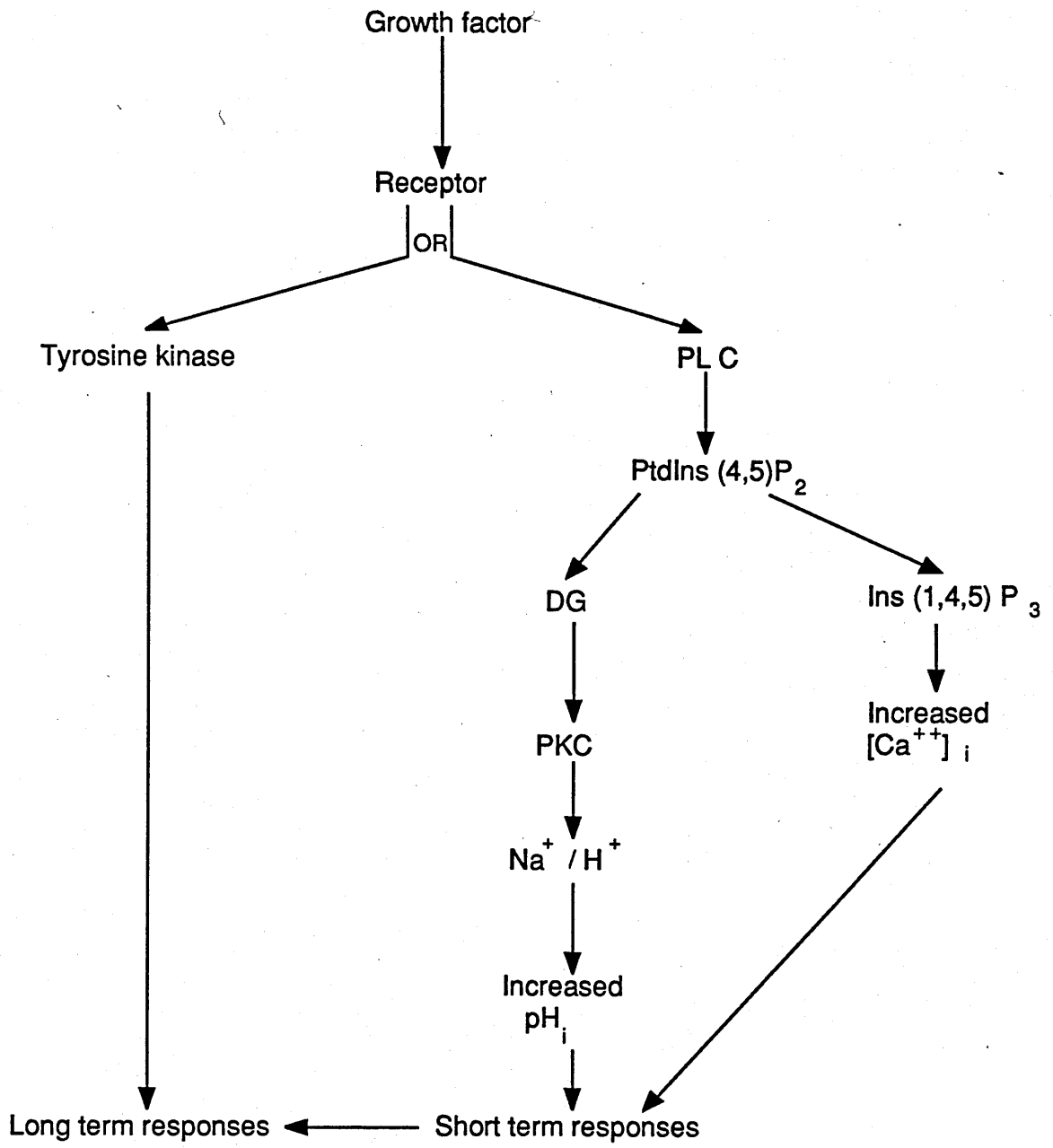
Increases in the metabolism of inositol lipids have therefore been associated with the mitogenesis of cells from several tissues where it is thought to be involved in the early events in the cellular processes that lead to proliferation and differentiation (Michell 1982; Berridge 1986 and 1987a). It is the changes in  $[Ca^{++}]_i$  concentration and  $pH_i$ , via the PKC activation of the  $Na^+/H^+$  antiporter, which act in synergy to bring about the obligatory steps involved in initiating DNA synthesis (Rozengurt 1986) and is summarised in Figure 3.1.

The importance of  $PtdIns(4,5)P_2$  hydrolysis in the stimulation of mitogenesis has been also demonstrated by Matuoka et al (1988). They showed that a monoclonal antibody to  $PtdIns(4,5)P_2$  abolished the mitogenic action of PDGF, a growth factor known to stimulate PPI turnover, on NIH 3T3 cells (Fukami et al 1988). This antibody had no effect on

Figure 3.1. Summary of events leading from growth factor-receptor binding to an increase in  $[Ca^{++}]_i$  and  $pH_i$ .



Fig. 3.1



the proliferative action of EGF, FGF or insulin, growth factors which do not stimulate PPI turnover in these cells.

As a link has been established between inositol lipid metabolism and cell division a review of this signalling system, outlining the major pathways involved in the metabolism of inositol phospholipids and inositol phosphates, is given in Figure 3.2. This figure is by no means exhaustive and more detailed information can be found in Irvine et al (1988) and Majerus et al (1988). The interaction of these proliferative agents with their respective receptor on their target cell initiates the hydrolysis of the minor membrane phospholipid  $\text{PtdIns}(4,5)\text{P}_2$  by PL C. The cell surface receptor is coupled to PL C via a G protein designated  $\text{G}_p$  (Cockcroft and Gomperts 1985; for reviews see Taylor and Merritt 1986 and Gilman 1987).  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis yields the two intracellular second messengers DG and  $\text{Ins}(1,4,5)\text{P}_3$  (for comprehensive reviews of this signal transduction pathway see Berridge 1984; Berridge and Irvine 1984; Hokin 1985; Abdel-Latif 1986; Downes 1986; Nahorski et al 1986; Berridge 1987b; Fisher and Agranoff 1987; Nishizuka 1988; Berridge and Irvine 1989).

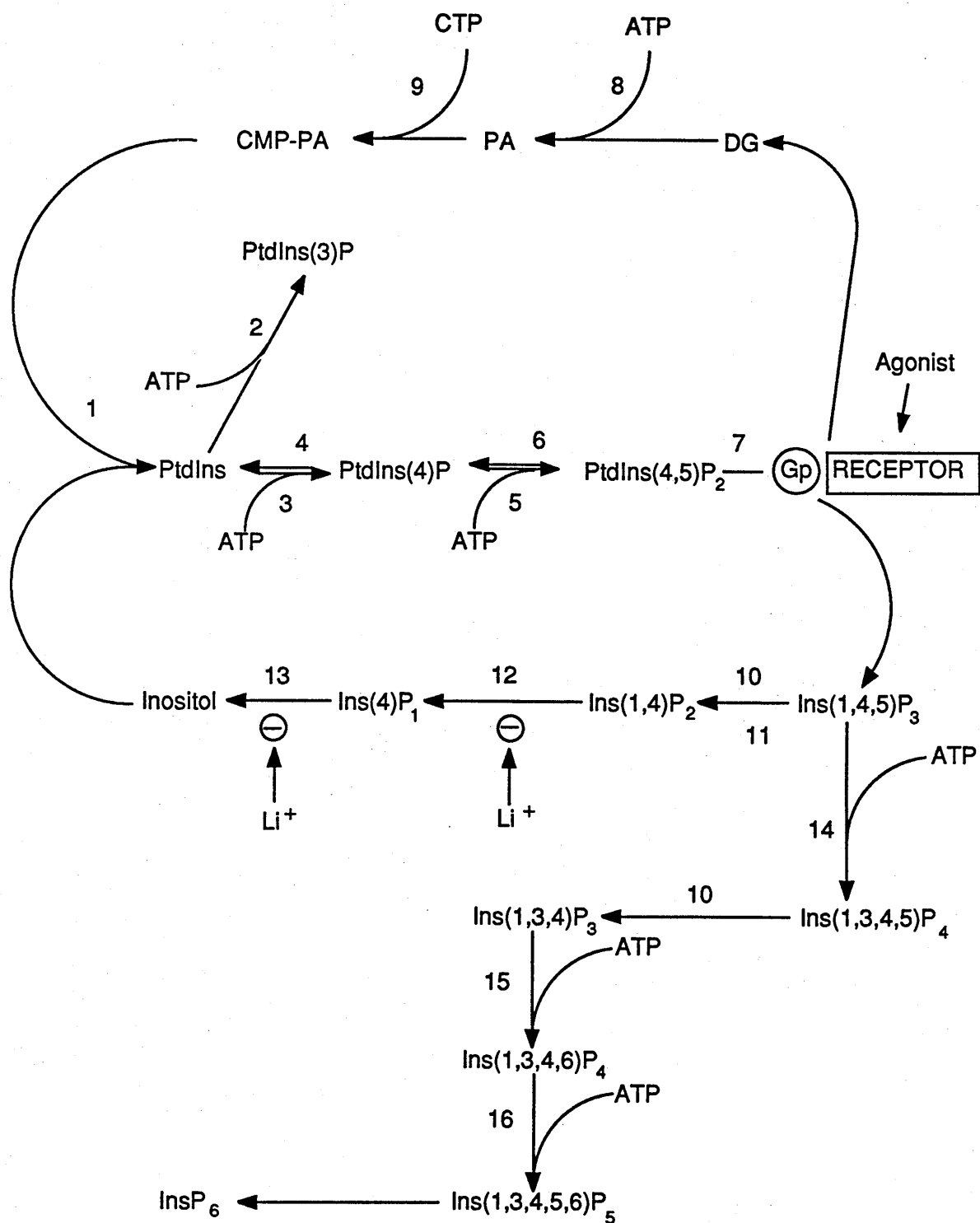
$\text{Ins}(1,4,5)\text{P}_3$  mobilises intracellular calcium from a non-mitochondrial pool (Streb et al 1983) by binding to high affinity receptors (Willcocks et al 1987) which are linked to a calcium channel (Berridge 1987b). The intracellular calcium pool has characteristics which suggest that it is

Figure 3.2. Major pathways of metabolism of inositol phospholipids and inositol phosphates.

Enzymes of major pathways

1. PtdIns synthetase
2. PtdIns-3-kinase (Type I)
3. PtdIns-4-kinase (Type II)
4. PtdIns(4)P phosphomonoesterase
5. PtdIns(4)P-5-kinase
6. PtdIns(4,5)P<sub>2</sub> phosphomonoesterase
7. Phospholipase C
8. DG kinase
9. CMP-PA synthetase
10. Ins(1,4,5)P<sub>3</sub>/Ins(1,3,4,5)P<sub>4</sub>-5-phosphatase
11. Ins(1,4,5)P<sub>3</sub>-5-phosphatase
12. Inositol polyphosphate-1-phosphatase
13. InsP phosphatase
14. Ins(1,4,5)P<sub>3</sub>-3-kinase
15. Ins(1,3,4)P<sub>3</sub>-6-kinase
16. Ins(1,3,4,6)P<sub>4</sub>-5-kinase

Fig. 3.2



the endoplasmic reticulum (ER) but only a part of it is Ins(1,4,5)P<sub>3</sub>-sensitive. Ins(1,4,5)P<sub>3</sub> receptors are localised on the nuclear envelope, and on parts of the ER near the nucleus. Recently, it has been suggested that a cytoplasmic organelle, that is not continuous with the ER but is close to it, called a "calciosome", which has characteristics of the sarcoplasmic reticulum, may be the Ins(1,4,5)P<sub>3</sub>-sensitive calcium pool (Volpe et al 1988). The locations of the Ins(1,4,5)P<sub>3</sub>-sensitive and -insensitive pools are therefore still uncertain and their relationship to calcsosomes is not yet clear (Berridge and Irvine 1989).

As can be seen from Figure 3.2. Ins(1,4,5)P<sub>3</sub> can be sequentially dephosphorylated to inositol (Downes et al 1982; Storey et al 1984) or phosphorylated to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P<sub>4</sub>) (Batty et al 1985; Irvine et al 1986b). Ins(1,3,4,5)P<sub>4</sub> has been proposed to act as an additional intracellular messenger (Irvine et al 1984b; Batty et al 1985). In association with Ins(1,4,5)P<sub>3</sub> it is thought to regulate the entry of calcium from the external medium (Irvine and Moor 1986 ; Morris et al 1987; Irvine 1989; Berridge and Irvine 1989). Changes in calcium entry from outside the cell may be important during long term processes like proliferation (Hesketh et al 1985).

More highly phosphorylated forms of inositol, inositol pentakisphosphate (InsP<sub>5</sub>) and inositol hexakisphosphate (InsP<sub>6</sub>) also exist. Until recently these had only been found in plants and avian red blood cells but now they have also been identified in animal cells (Heslop et al 1985).

There is little evidence of association of  $\text{InsP}_5$  or  $\text{InsP}_6$  with receptor-activated PPI turnover (Heslop et al 1985; Nahorski and Batty 1986), although they have been proposed to act as a phosphate store (Irvine et al 1988). A role as extracellular messengers has however been proposed by Vallejo et al (1987). An enzyme that is capable of hydrolysing  $\text{InsP}_5$  has been found on the extracellular surface of NIH 3T3 cells (Carpenter et al 1989).

DG activates the calcium- and phosphatidylserine-dependent PKC, which phosphorylates serine and threonine residues on its target proteins (Nishizuka 1984). DG can also be released by the  $\text{Ca}^{++}$ -dependent action of another PL C, on phosphatidylinositol 4-phosphate ( $\text{PtdIns}(4)\text{P}$ ) and  $\text{PtdIns}$ , which has been reported in platelets (Majerus et al 1985). DG is metabolised either via a DG lipase to yield arachidonic acid which is used as a precursor for prostaglandins and leukotrienes (Irvine 1982) or is phosphorylated by DG kinase to PA. This latter pathway seems to be the predominant route used by platelets (Billah et al 1981) and in brain (Van Rooijen et al 1985; Dudley and Spector 1986).

It was Michell (1975) who suggested that increased inositol phospholipid metabolism may be responsible for the receptor-stimulated increase in  $[\text{Ca}^{++}]_i$  that initiates a number of physiological responses in cells. It was thought that the initial reaction involved  $\text{PtdIns}$  metabolism which was measured by increased  $^{32}\text{P}$  labelling of  $\text{PtdIns}$  and PA. PA, which is formed during receptor-activated  $\text{PtdIns}$  breakdown,

was thought to be responsible for the changes that were seen in  $[Ca^{++}]_i$  since it was seen to accumulate in response to calcium mobilising stimuli (Lapetina and Cuatrecasas 1979; Putney et al 1980; Salmon and Honeyman 1980). PA also provoked calcium translocation across an organic phase in a Pressman cell (Tyson et al 1976) and into liposomes (Serhan et al 1981 and 1982; Blau and Weissmann 1988). It was proposed that PA acted as a calcium ionophore to bring about the physiological effects of these stimuli in a variety of different cell types (Barritt et al 1981; Harris et al 1981; Ohsako and Deguchi 1981 and 1983). Controversy did exist however about the calcium ionophore hypothesis of PA action since Holmes and Yoss (1983) showed that PA was unable to translocate calcium across PC membranes.

It is now known that  $PtdIns(4,5)P_2$  is the major phospholipid hydrolysed (Michell et al 1981), and that  $Ins(1,4,5)P_3$  is the intracellular messenger formed during PPI turnover that is responsible for calcium mobilisation. This put an end to the calcium ionophore hypothesis of PA. However, recent work on the growth factor-like action of PA on fibroblasts has renewed interest in PA as an intracellular signal molecule (Moolenaar et al 1986; Siegmann 1987; Yu et al 1988).

PA has been found to stimulate PPI turnover in fibroblasts and that this was not due to its acting as an ionophore since the effects were independent of extracellular calcium (Moolenaar et al 1986; Murayama and Ui 1987a). PA-stimulated PL C hydrolysis of  $PtdIns(4,5)P_2$  in platelet

membranes has also been reported (Jackowski and Rock 1989). The effect of PA was dependent on the presence of longer chain length fatty acids, for example, palmitic ( $C_{16:0}$ ) or stearic acid ( $C_{18:0}$ ) at  $R_1$  and arachidonic acid ( $C_{20:4}$ ) at  $R_2$  (see Figure 3.3.), similar to those found in the endogenous molecule, rather than lauric acid ( $C_{12:0}$ ) at both  $R_1$  and  $R_2$  (Moolenaar et al 1986; Murayama and Ui 1987a). PA has also been reported to increase  $[Ca^{++}]_i$  (Moolenaar et al 1986; Kawase and Suzuki 1988). PA is thought to bring about changes in  $[Ca^{++}]_i$  by stimulating PPI turnover to produce  $Ins(1,4,5)P_3$  (Moolenaar et al 1986).

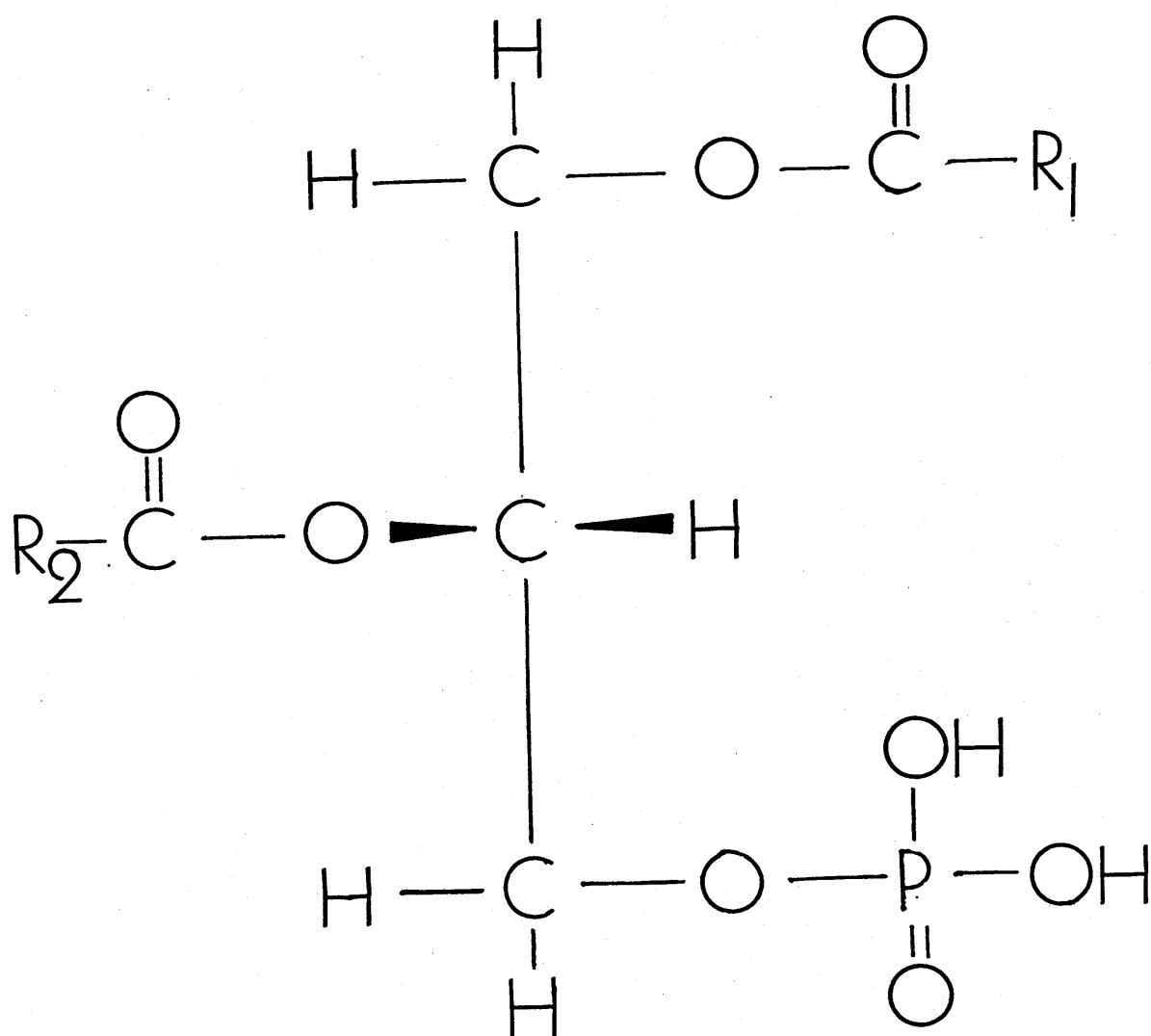
Astrocytes have been found to possess a variety of receptor types which are coupled to PPI turnover, for example  $\alpha_1$ -adrenergic, muscarinic cholinergic (Pearce et al 1985, Pearce et al 1986a and Gonzales et al 1985), histamine, 5-hydroxytryptamine (Pearce et al 1985; Arbones et al 1988), quisqualate-preferring glutamate (Pearce et al 1986b),  $P_2$ -purinergic (Pearce et al 1989), angiotensin II receptors (Raizada et al 1987) and receptors for a number of peptides (Cholewinski and Wilkin 1988; Cholewinski et al 1988) (for reviews see Murphy and Pearce 1987 and Pearce and Murphy 1988). Could PA also be activating PPI turnover in astrocytes to bring about its mitogenic action?

The ability of agonists to stimulate inositol phospholipid hydrolysis can be studied by monitoring the accumulation of the intracellular metabolites of inositol phospholipid breakdown. This was made possible by the observation that the myo-inositol level of the cerebral cortex of rats



Figure 3.3. Stereochemical configuration of PA showing the stereospecific numbering of the fatty acid side chains attached to the carbon atoms.

Figure 3.3.



treated with lithium was decreased, with an associated increase in the myo-inositol 1-phosphate content, due to the inhibition by lithium of the enzyme myo-inositol 1-phosphatase (Allison et al 1976). The addition of lithium can thus be used to amplify the agonist-dependent PPI turnover responses of a variety of cell types (Berridge et al 1982). The separation of the inositol phosphates is affected by anion exchange chromatography and that of the inositol phospholipids by thin layer chromatography (TLC).

The action of PA on inositol phosphate accumulation and inositol phospholipid labelling in astrocytes and  $^{45}\text{Ca}^{++}$  efflux from astrocytes was investigated. The responses of fibroblasts to PA were also determined.

In the remainder of this chapter I shall present evidence to show that:

(i) PA increased the accumulation of total and individual inositol phosphates in astrocytes and that this effect was dependent on extracellular calcium and the presence of PA containing long chain fatty acids.

(ii) PA reduced the labelling of  $\text{PtdIns}(4,5)\text{P}_2$  at short time points and after 5 minutes reduced the labelling of  $\text{PtdIns}$  and increased that of  $\text{PtdIns}(4)\text{P}$  and  $\text{PtdIns}(4,5)\text{P}_2$ . PA increased  $^{45}\text{Ca}^{++}$  efflux from astrocytes. Both these effects were dependent on the presence of PA containing long chain fatty acids.

(iii) PA had no detectable effect on PPI turnover in fibroblasts but did stimulate  $^{45}\text{Ca}^{++}$  efflux.

### 3.2. METHODS AND MATERIALS.

#### 3.2.1. Astrocyte Cultures.

Astrocyte-enriched cultures were prepared as described in Chapter 2 section 2.2.1. and grown in either 60mm diameter dishes or 6 well plates (35mm diameter).

#### 3.2.2. Phosphoinositide metabolism.

##### 3.2.2.1. Labelling.

Astrocyte-enriched cultures, 18-22 div, were labelled with [<sup>3</sup>H]myo-inositol (15 Ci/mmol). The GM was removed from each culture dish or well and 2ml. of labelled GM (1  $\mu$ Ci/ml) was added. The final concentration of inositol was 10  $\mu$ M. The cultures were incubated at 37°C in a 5% CO<sub>2</sub> /95% air atmosphere for either 24, 48, or 72 hours.

##### 3.2.2.2. Incubations.

After labelling, the medium was removed and the cultures washed twice with PSS (for composition see Chapter 2 section 2.2.2.1). 2ml. of PSS was then added to each dish or well and the cultures returned to the incubator for 45 minutes. The cultures were then washed twice and 2ml of fresh PSS added, supplemented with lithium chloride and calcium chloride, to a final concentration of 5 and 2.5mM respectively. For short time point incubations where changes in Ins(1,4,5)P<sub>3</sub> were being studied lithium chloride

was omitted since lithium chloride has been found to inhibit the formation of  $\text{Ins}(1,4,5)\text{P}_3$  (Batty and Nahorski 1985). In experiments where the calcium conditions were varied additional calcium chloride was either omitted, or it was omitted and ethyleneglycol-bis-(b-aminoethyl ether) N,N'-tetraacetic acid (EGTA) added to a final concentration of 0.5mM. The cultures were incubated for 15 minutes in the presence of lithium and calcium prior to the appropriate agonist addition being made. For some incubations lithium was added at the same time as agonist and calcium was already present in the PSS. After incubation for the appropriate length of time the incubations were stopped by removing the incubation medium, washing twice with PSS and adding 1ml. of  $\text{CHCl}_3$ :MeOH mixture (1:2 v/v) to each dish or well. The cells were scraped from the culture dish or well and transferred to test-tubes. 0.3ml of  $\text{CHCl}_3$  and 0.3ml of deionised water were added to each tube and after thorough mixing the phases were separated by centrifugation at 180g for 10 minutes at 4°C. An aliquot of the lower chloroform layer was removed, dried and 1ml of  $\text{H}_2\text{O}$  and 8ml of Cocktail T or Emulsifier Safe scintillant added for liquid scintillation counting of labelled inositol lipids.

#### 3.2.2.3. Measurement of [ $^3\text{H}$ ]inositol phosphates accumulation.

A 0.75ml. aliquot of the upper aqueous phase was taken and diluted with 2ml. deionised water for separation of inositol phosphates (IPs) by ion exchange chromatography as described by Berridge et al (1982). 0.5ml of a 50% v/v suspension of

Dowex ion exchange resin in the formate form was added to the IPs mixture. After mixing the suspension was transferred to a column and allowed to settle. Free [ $^3\text{H}$ ]myo- inositol was eluted by washing the column with five 2ml. volumes of 5mM myo-inositol and glycerophosphoinositol (GPI) and the [ $^3\text{H}$ ]-labelled IPs were eluted with 1ml. of 1M ammonium formate in 0.1M formic acid and collected for liquid scintillation counting. In some experiments GPI was eluted before the IPs by washing with five 2ml aliquots of 5mM borax, 60mM sodium formate and the IPs collected by elution with 1ml of 1M ammonium formate in 0.1M formic acid.

Separations of the individual IPs formed were also carried out, but for these experiments the aqueous extracts from 4 culture dishes or the 6 wells from a 6 well plate were pooled and diluted appropriately. A 1ml. aliquot of a 50% v/v suspension of formate form Dowex resin was added and after mixing the suspension was transferred to a column. This simple chromatographic technique does not allow the separation of the individual inositol phosphate isomers, for example Ins(1,3,4) $\text{P}_3$  and Ins(1,4,5) $\text{P}_3$ , and for this reason the individual inositol phosphates are referred to according to the number of phosphates present with no designation of position of those phosphate groups. The column was washed with four 2ml. aliquots of 5mM myo-inositol and the eluted [ $^3\text{H}$ ]myo-inositol discarded. The last myo-inositol wash was collected for liquid scintillation counting. The individual IPs were eluted with formate solutions of increasing strength according to the method of Brown et al (1984). GPI was eluted with five 2ml.

aliquots of 5mM borax, 60mM sodium formate.  $\text{InsP}_1$  was eluted with eight 2ml. aliquots of 5mM borax, 150mM sodium formate.  $\text{InsP}_2$  was eluted with five 2ml. aliquots of 0.3M ammonium formate in 0.1M formic acid.  $\text{InsP}_3$  was eluted with three 2ml. aliquots of 0.75M ammonium formate in 0.1M formic acid. Each 2ml. aliquot was collected and 10ml of scintillant added for the determination of the [ $^3\text{H}$ ]inositol content of each IP fraction by liquid scintillation counting.

#### 3.2.2.4. Expression of results.

The radioactivity recovered in IPs was corrected to a standard incorporation of radiolabel ( $10^5$  dpm) into the inositol lipids of each culture. The incorporation of label into inositol lipids was between 85,000 and 130,000 dpm for 60mm diameter dishes and between 45,000 and 65,000 dpm for 35mm diameter wells from a 6 well plate. The radioactivity recovered in IPs where individual IPs from 4 culture dishes or a 6 well plate were examined was also corrected to a standard incorporation of radiolabel ( $10^6$  dpm) into the inositol lipids. The incorporation into inositol lipids from 4 culture dishes and a 6 well plate was approximately 400,000 dpm. This normalisation was carried out to allow comparison of results from experiments using different batches of cells.



### 3.2.2.5. [<sup>3</sup>H]Inositol phospholipid composition.

Determination of the labelled inositol phospholipid composition of astrocyte cultures was carried out by TLC according to the method of Jolles et al (1981). An aliquot of the CHCl<sub>3</sub> layer obtained from the PPI turnover experiments containing the labelled inositol phospholipids was taken and dried. High performance silica gel 60F TLC plates, treated with 1% w/v potassium oxalate in MeOH:H<sub>2</sub>O (2:3 v/v) were activated by heating at 110°C for 15 minutes. Each lipid sample was resuspended in an aliquot of CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O (75:25:2 v/v) for application to the TLC plate. A phospholipids standard mixture containing 5 µg, of the sodium salt in CHCl<sub>3</sub>, of each of the following phospholipids: PA, PtdIns, PtdIns(4)P and PtdIns(4,5)P<sub>2</sub> was run on each plate. Separation was achieved by a mobile phase containing CHCl<sub>3</sub>:acetone:MeOH:acetic acid:H<sub>2</sub>O (40:15:13:12:8 v/v). The following R<sub>f</sub> values were obtained for these phospholipid standards: PA 0.93; PtdIns 0.53; PtdIns(4)P 0.36 and PtdIns(4,5)P<sub>2</sub> 0.29. The phospholipids were visualised by exposure to iodine vapour. The spots were scraped, 0.5ml H<sub>2</sub>O and 8ml of scintillant added and the [<sup>3</sup>H]inositol content of the inositol phospholipids determined by liquid scintillation counting.

### 3.2.3. Measurement of <sup>45</sup>Ca<sup>++</sup> efflux

#### 3.2.3.1. Labelling and Incubations

Calcium efflux was determined by using an adaptation of the

method of Lazarewicz and Kanje (1981). Cells grown for 18-22 div in 6 well plates were labelled in GM containing  $2 \mu\text{Ci ml}^{-1}$   $^{45}\text{Ca}^{++}$  for 24h. After incubation for 5 or 10 minutes in a pre-gassed PSS of the following composition (in mM): NaCl 116,  $\text{NaHCO}_3$  26.2, Glucose 5.5, KCl 5,  $\text{MgSO}_4$  1.5,  $\text{CaCl}_2$  1.3 and  $\text{NaH}_2\text{PO}_4$  1, cells were incubated at room temperature in 2ml aliquots of PSS for three or four 1 minute intervals to establish basal  $^{45}\text{Ca}^{++}$  efflux. Agonist was then added at the appropriate concentration to the cells in 2ml PSS for 1 minute and  $^{45}\text{Ca}^{++}$  efflux into this aliquot monitored. Post agonist addition efflux into four further 2ml PSS aliquots was also monitored. The cells were then solubilised by adding 1ml 0.1M NaOH to each well to allow the determination of the amount of radiolabel remaining in the cells. After 10 minutes the cells were scraped from the well. Each well was then washed with 1ml  $\text{H}_2\text{O}$  and this was added to the NaOH aliquot. The  $^{45}\text{Ca}^{++}$  content of the PSS aliquots and the solubilised cell suspension was determined by liquid scintillation counting.

#### 3.2.3.2. Expression of results

$^{45}\text{Ca}^{++}$  efflux was calculated as the  $^{45}\text{Ca}^{++}$  released per minute as a percentage of that remaining in the cell at that time. The effect of a 1 minute incubation with PA on  $^{45}\text{Ca}^{++}$  efflux was expressed as a percentage of the efflux prior to the addition of PA.

#### 3.2.4. Protein content

Growth medium was removed and the cells rinsed twice with PSS. 1ml H<sub>2</sub>O was added to each dish or well and the cells removed by scraping. The protein content of an aliquot of the astrocyte suspension was determined by using the method of Lowry et al (1951) using BSA as the standard.

#### 3.2.5. Fibroblast cultures.

The meninges from 12 neonatal rat cerebral cortices were pooled and incubated in disaggregation medium containing 2.5 mgml<sup>-1</sup> collagenase for 15 minutes at 37°C in a shaking water bath. The cells were then mechanically dissociated by trituration through a 1.5mm diameter stainless steel cannula. The cell suspension was diluted in growth medium, plated onto 6 well (35mm diameter well) plates and grown in a humid 5% CO<sub>2</sub>/95% air atmosphere with medium changes every 3 or 4 days. Confluent cultures were obtained after 14-18 div.

#### 3.2.6. Statistical analysis

Statistical analysis was carried out using the Mann-Whitney U test. Students unpaired t-test was used for the experiments where individual inositol phosphates were determined. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01, \*\*\*\* 0.005.

### 3.2.7. Materials

Collagenase, PA from egg yolk lecithin, the sodium salts of PA containing different chain length fatty acids, PtdIns from soybean, PtdIns(4)P and PtdIns(4,5)P<sub>2</sub> from bovine brain were obtained from Sigma. PA as the free acid in CHCl<sub>3</sub>:MeOH was obtained from Lipid Products, Surrey and PA as the ammonium salt in CHCl<sub>3</sub>:MeOH was obtained from Serdary Research Labs. Inc., Canada. Ion exchange chromatography materials were obtained from Sigma and BDH. Lithium chloride, the TLC solvents, potassium oxalate and iodine were obtained from BDH. High performance silica gel 60F TLC plates were obtained from Merck. Myo-[2-<sup>3</sup>H]inositol (water:ethanol 9:1 v/v) 15Ci/mmol and <sup>45</sup>calcium chloride in 0.15M HCl (15 mCi/mg calcium) were obtained from Amersham International PLC.

### 3.3. RESULTS.

3.3.1. The effect of PA on the accumulation of [ $^3\text{H}$ ]inositol phosphates in astrocytes.

#### 3.3.1.1. Concentration dependence

Astrocyte-enriched cultures that had been prelabelled for 24h with [ $^3\text{H}$ ]myo-inositol were exposed to various concentrations of PA for 30 minutes in the presence of lithium chloride (5mM). The addition of PA caused a dose-dependent increase in IPs accumulation in the concentration range 10 to 500  $\mu\text{gml}^{-1}$  (Figure 3.4. (exp 1)). 0.1 and 1  $\mu\text{gml}^{-1}$  PA were without effect. At 50  $\mu\text{gml}^{-1}$  PA (71  $\mu\text{M}$ ) maximal stimulation of 183% of control was seen, giving an  $\text{EC}_{50}$  of 25  $\mu\text{gml}^{-1}$  (36  $\mu\text{M}$ ).

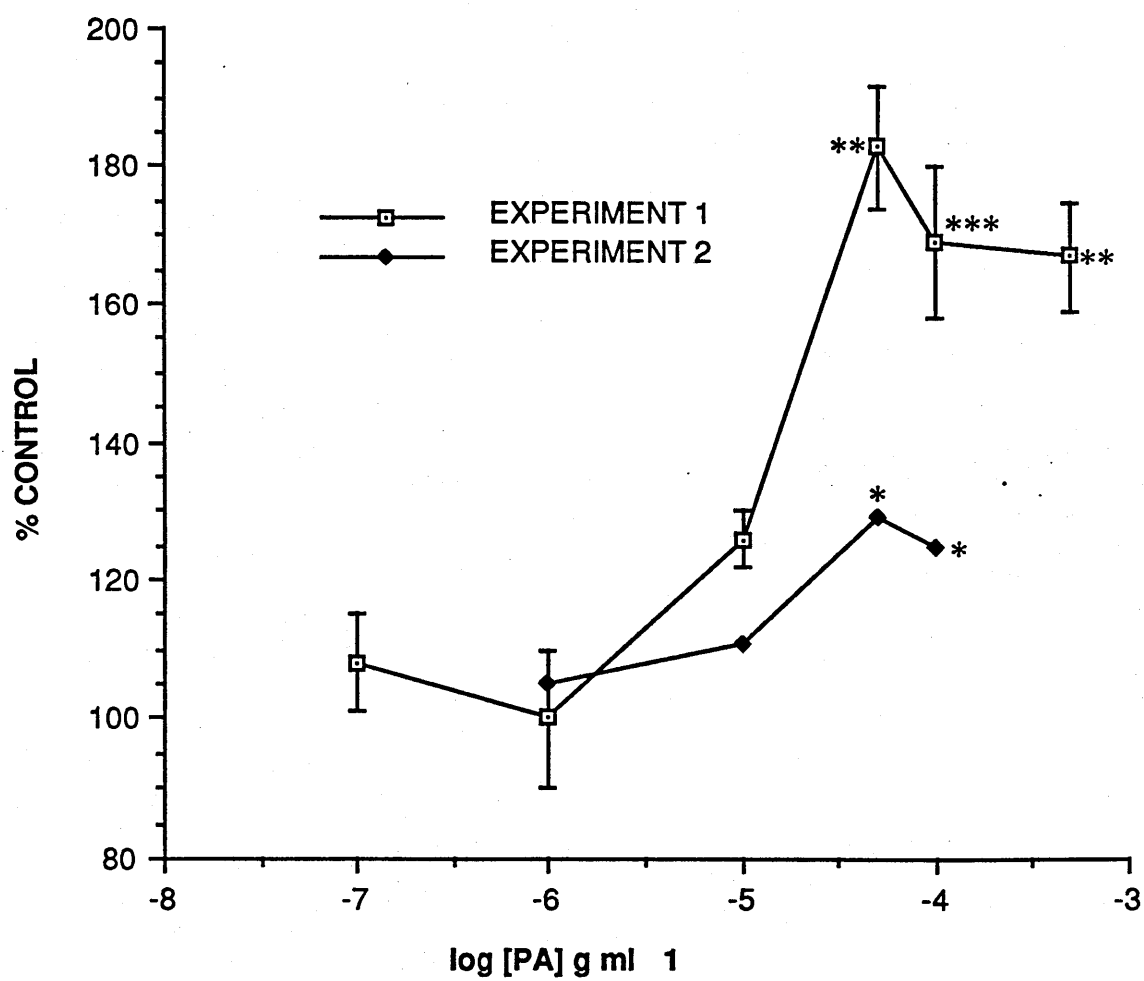
In a similar experiment where the effects of 1, 10, 50 and 100  $\mu\text{gml}^{-1}$  PA were investigated (Figure 3.4., exp 2), 10  $\mu\text{gml}^{-1}$  was this time without effect but maximal stimulation was again seen at 50  $\mu\text{gml}^{-1}$  but the increase was only 129% of control compared to 183% for the previous experiment. The dose response curve did however give an  $\text{EC}_{50}$  similar to that obtained in the previous experiment. Thus an increase in PPI turnover was seen in astrocytes in response to PA but this response was found to be variable.

When the data from this experiment (Figure 3.4. exp 1) is plotted with that obtained by Murphy and Pearce (1988; also Pearce and Murphy 1988) for the effect of PA on

Figure 3.4. The concentration dependence of PA-induced [<sup>3</sup>H]inositol phosphates accumulation (GPI and IPs).

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and then exposed to various concentrations of PA (in the presence of 5mM lithium chloride) for 30 minutes. Results are mean +/- SEM (n = 6 for exp 1 and n = 3 for exp 2) using 60mm dishes and are expressed as % of control incubations (no PA added). Mean dpm for control incubations = 1150 dpm for n = 6 and 723 dpm for n = 3. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to control incubation (no PA added).

FIG. 3.4



[<sup>3</sup>H]thymidine incorporation into astrocyte DNA (Figure 3.5.) it can be seen that PA shows the same efficacy for [<sup>3</sup>H]IPs accumulation and DNA synthesis.

PA obtained from Sigma was initially dry and powdery but on storage it did take on a more waxy appearance. The source of the variable response in astrocytes may therefore have been due to the state of the PA that was used. This was investigated by comparing the effect of freshly opened PA to that which had been stored. The experiment was carried out as outlined above with prelabelled astrocytes except that the cells were exposed to two different sorts of PA at 50  $\mu\text{gml}^{-1}$  for 30 minutes and the IPs accumulated determined. As can be seen from Table 3.1. both types of PA caused a significant increase in IPs accumulated but that in response to dry PA was greater than that seen in response to waxy PA, 152% of control compared to 129% of control respectively. The variable response of astrocytes to PA may therefore have been due to a variation in the state of the PA used in different experiments, although a response was still seen when PA of a more waxy appearance was used. In an attempt to overcome this variation in response to the phospholipid, PA was stored as a 10  $\text{mgml}^{-1}$  stock in  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) in the freezer and diluted appropriately for use.

#### 3.3.1.2. Time Course.

Prelabelled astrocyte-enriched cultures were exposed to 50  $\mu\text{gml}^{-1}$  PA, diluted from the 10  $\text{mgml}^{-1}$  stock for varying lengths of time. The control incubations were stopped after



Figure 3.5. The relationship between PA-induced [ $^3$ H]inositol phosphates accumulation (GPI and IPs) and PA-stimulated [ $^3$ H]thymidine incorporation.

[ $^3$ H]Inositol phosphates accumulation (GPI and IPs) see Figure 3.4. legend.

[ $^3$ H]Thymidine incorporation. Subconfluent serum-depleted cultures were exposed to various concentrations of PA for 24h. Results are mean  $\pm$  SEM (n = 4) using 24 well plates and are expressed as % of 0.1% FCS GM control.

FIG. 3.5

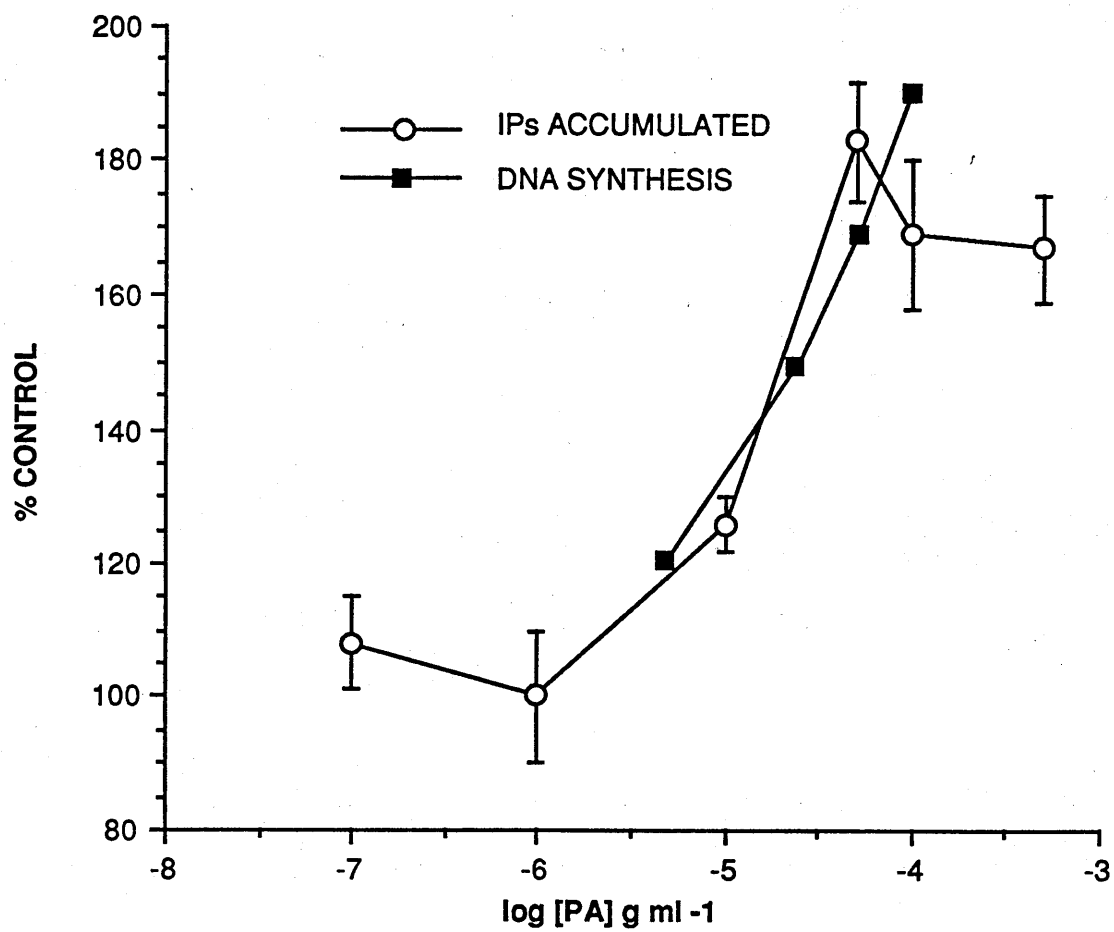


Table 3.1. The effect of PA in different conditions on the accumulation of [<sup>3</sup>H]inositol phosphate (GPI and IPs).

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and then exposed to either dry or waxy PA at 50  $\mu\text{gml}^{-1}$  for 30 minutes (in the presence of 5mM lithium chloride). Results are mean +/- SEM (n = 8) using 60mm dishes. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to control incubation (no PA added).

Sample	Normalised dpm	% control	p level
Control	918 +/- 71	100 +/- 8	
PA waxy	1182 +/- 73	129 +/- 8	**
PA dry	1395 +/- 97	152 +/- 11	***

the lithium preincubation. After 5 minutes exposure to 50  $\mu\text{gml}^{-1}$  PA the increase in IPs accumulation was 224% of control (Figure 3.6.). The response then declined to only 122% of control by 60 minutes.

The individual IPs formed by prelabelled astrocyte-enriched cultures in response to 50  $\mu\text{gml}^{-1}$  PA for 5 and 30 minutes were also examined (Table 3.2.). After 5 minutes exposure to 50  $\mu\text{gml}^{-1}$  PA increases were seen in the accumulation of  $\text{InsP}_1$  and  $\text{InsP}_2$  of 280% and 422% of control respectively. An increase of 146% of control was also seen in the  $\text{InsP}_3$  fraction at this shorter time point. After 30 minutes exposure an increase in  $\text{InsP}_1$  of 241% of control was detected but increases were no longer seen in the  $\text{InsP}_2$  and  $\text{InsP}_3$  fractions.

The time course experiments outlined in Figure 3.6. and Table 3.2. were carried out using the PA stock in  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) which was diluted appropriately for use. The  $\text{CHCl}_3:\text{MeOH}$  vehicle itself however, was found to stimulate PPI turnover in astrocytes. A preliminary experiment was carried out to investigate the effect of  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) in the concentration range 0.01 to 1.0% v/v on IPs accumulation after 30 minutes exposure. 0.5 and 1.0% v/v  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) caused significant increases in accumulation of 189% and 382% of control respectively (Figure 3.7.).

The time course experiment that was carried out to determine the total IPs accumulation in response to 50  $\mu\text{gml}^{-1}$  PA had

Figure 3.6. The time course of PA-induced [ $^3\text{H}$ ]inositol phosphate accumulation (GPI and IPs).

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and exposed to PA at  $50\text{ }\mu\text{gml}^{-1}$  for the times indicated (in the presence of 5mM lithium chloride). Results are mean  $\pm$  SEM (n = 3) using 60mm dishes and are expressed as % of control incubations (stopped after lithium preincubation). Mean dpm for control incubations = 1305 dpm. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to control incubation (stopped after lithium preincubation).

FIG. 3.6

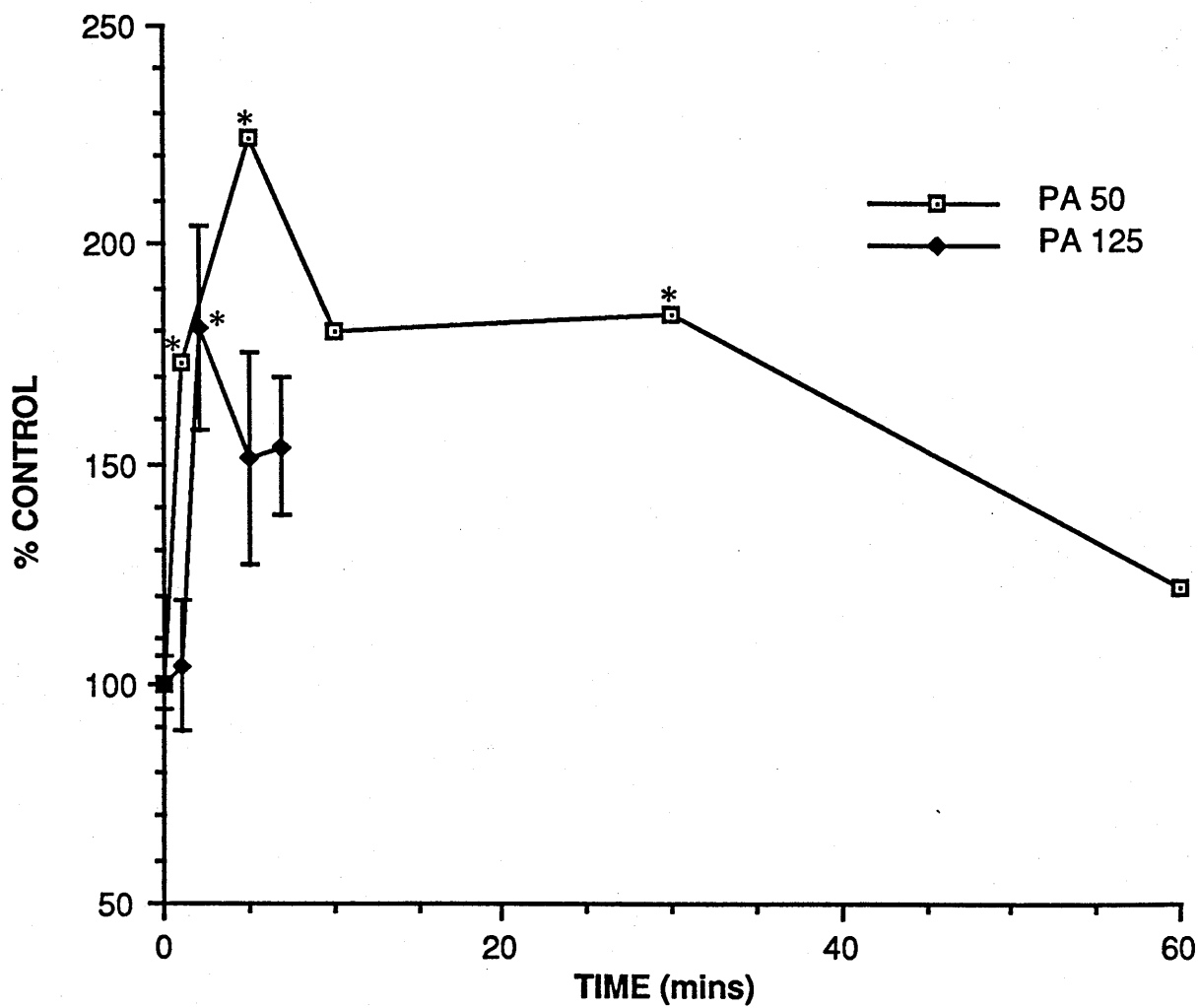


Table 3.2. PA-induced individual [ $^3\text{H}$ ]inositol phosphate accumulation.

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and then exposed to PA at  $50\mu\text{gml}^{-1}$  for 5 or 30 minutes (in the presence of 5mM lithium chloride).

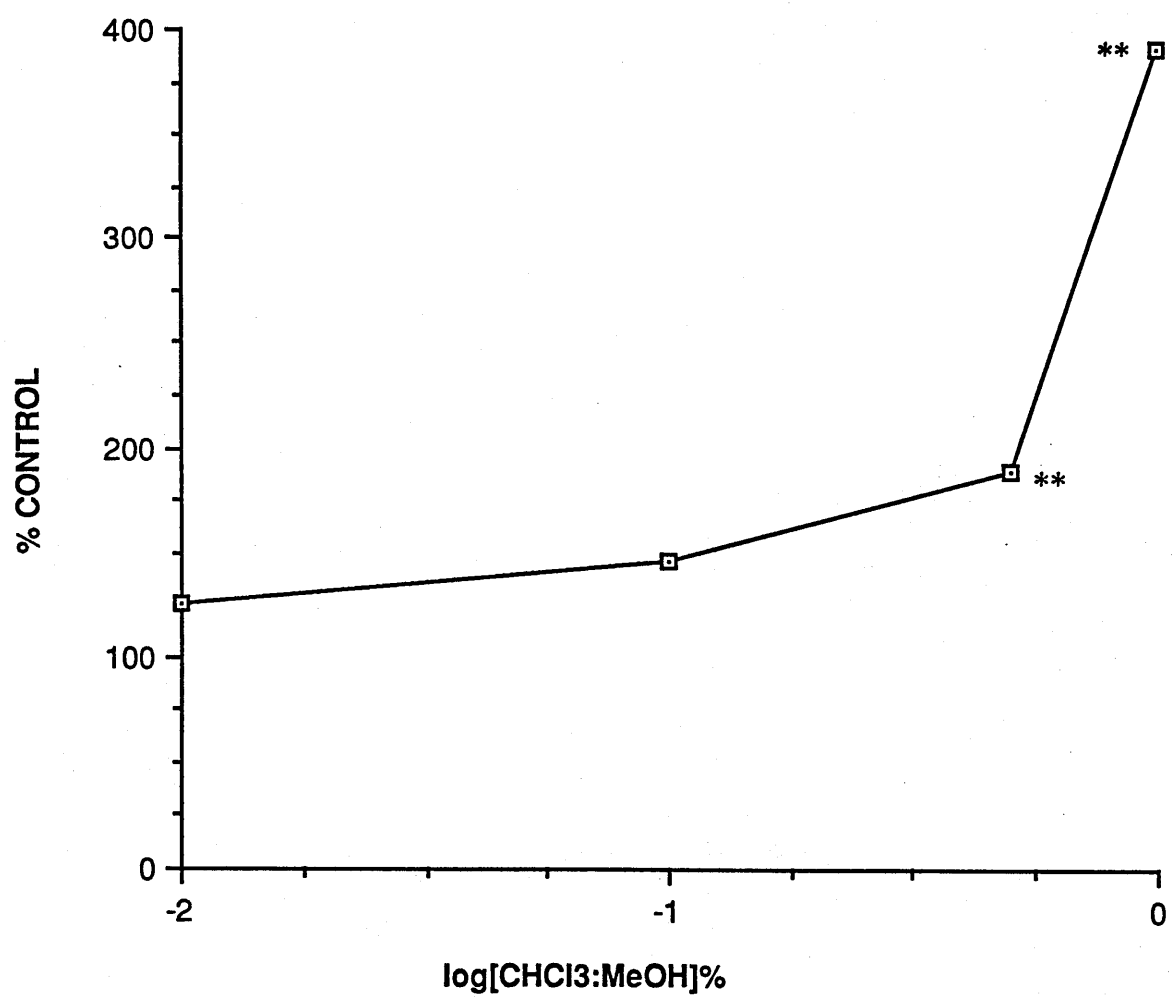
IP fraction	Total dpm/IP fraction			
	Time (mins)			
	5		30	
	Control	PA	Control	PA
GPI	8185	5448	5197	7604
InsP <sub>1</sub>	4986	13979	7806	18847
InsP <sub>2</sub>	3110	13120	8281	9138
InsP <sub>3</sub>	985	1437	1418	917
Total	17266	33984	22702	36506

Figure 3.7. The effect of various concentrations of  $\text{CHCl}_3$ :MeOH (1:2 v/v) mixture on  $[\text{}^3\text{H}]$ inositol phosphate accumulation (GPI and IPs).

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and then exposed to various concentrations of  $\text{CHCl}_3$ :MeOH (1:2 v/v) for 30 minutes (in the presence of 5mM lithium choride). Results are mean  $\pm$  SEM (n = 3) using 60mm dishes and are expressed as % of control incubations (no  $\text{CHCl}_3$ :MeOH added). Mean dpm for control incubations = 1000 dpm. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to control incubation (no  $\text{CHCl}_3$ :MeOH added).



FIG. 3.7



0.5%  $\text{CHCl}_3$ :MeOH (1:2 v/v) present. The increases seen in IPs accumulation may consequently have been due to the presence of  $\text{CHCl}_3$ :MeOH (1:2 v/v). Exposure for longer time periods, for example up to 60 minutes, may have had a detrimental effect on the cultures. Material was seen to have lifted off the dishes after these longer time periods and a decrease in the recovery of membrane inositol lipids of up to 18% was seen with increasing  $\text{CHCl}_3$ :MeOH (1:2 v/v) concentration (Table 3.3.).

The effect of PA in a non-stimulatory concentration of  $\text{CHCl}_3$ :MeOH (1:2 v/v) on the accumulation of individual IPs was therefore investigated to check that PPI turnover was being seen in response to PA. Cells that had been prelabelled for 72h in this instance were exposed to PA at  $100 \mu\text{gml}^{-1}$  in 0.1%  $\text{CHCl}_3$ :MeOH (1:2 v/v) for 1 minute in the absence of lithium and compared to control incubations that had 0.1%  $\text{CHCl}_3$ :MeOH (1:2 v/v) added to them. PA still caused an increase in the accumulation of  $\text{InsP}_3$  of 170% of control after 1 minute (Table 3.4.). A representative elution profile of the ion exchange chromatography of the individual IPs is shown in Figure 3.8.

The accumulation of total IPs by prelabelled astrocyte-enriched cultures at various time points in response to  $50 \mu\text{gml}^{-1}$  PA that contained no solvent, aqueous PA, was also examined. PA was prepared from a  $10 \text{ mgml}^{-1}$  stock in  $\text{CHCl}_3$ :MeOH (1:2 v/v) from which the solvent was removed by  $\text{N}_2$  and the PA sonicated in PSS. An increase in accumulation of 136% of control was seen by 5 minutes which was

Table 3.3. The effect of  $\text{CHCl}_3$ :MeOH (1:2 v/v) on  $[^3\text{H}]$ inositol phospholipid recovery.

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and then exposed to various concentrations of  $\text{CHCl}_3$ :MeOH (1:2 v/v) for 30 minutes (in the presence of 5mM lithium chloride). Results are mean  $\pm$  SEM (n = 3) using 60mm dishes. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to control incubation (no  $\text{CHCl}_3$ :MeOH added).

% Concentration of $\text{CHCl}_3$ :MeOH (1:2 v/v)	Dpm in lipids	p level
0	117990 $\pm$ 2476	
0.01	111654 $\pm$ 1494	
0.10	105478 $\pm$ 5398	
0.50	103129 $\pm$ 1627	**
1.00	96792 $\pm$ 561	**

Table 3.4. The effect of PA in 0.1% CHCl<sub>3</sub>:MeOH (1:2 v/v) on the accumulation of individual [<sup>3</sup>H]inositol phosphates.

Astrocyte-enriched cultures were prelabelled for 72h and then exposed to PA at 100  $\mu$ gml<sup>-1</sup> in 0.1% CHCl<sub>3</sub>:MeOH (1:2 v/v) for 1 minute (in the absence of lithium chloride).

Results are mean +/- SEM (n = 3) using 60mm dishes.

Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each IP fraction compared to appropriate control IP fraction.

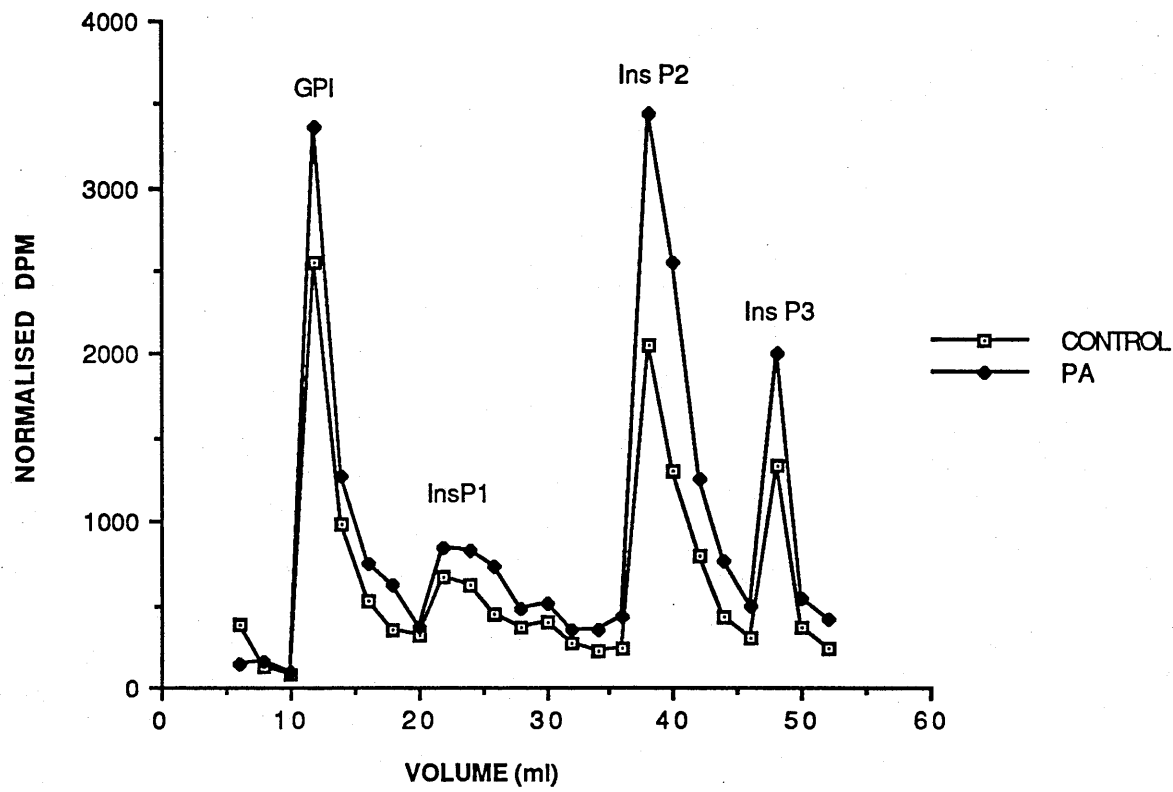
Total normalised dpm/IP fraction

Sample	InsP <sub>1</sub>	InsP <sub>2</sub>	InsP <sub>3</sub>	p level
Control	2629 +/-243	3160 +/-421	559 +/- 59	
PA	2361 +/- 92	2978 +/-192	953 +/-152	*

Figure 3.8. Representative elution profiles of the ion exchange chromatography of [<sup>3</sup>H]inositol phosphates.

Astrocyte-enriched cultures prelabelled for 24h were exposed to 50  $\mu\text{gml}^{-1}$  PA for 1 minute (in the absence of lithium). The aqueous phase from the pooled cultures was applied to the column and the [<sup>3</sup>H]inositol phosphates eluted as follows: 4x 2ml. inositol 5mM washes discarded and 1x2ml. inositol wash collected; 5x2ml. 5mM borax, 60mM sodium formate to elute GPI; 8x2ml. 5mM borax, 150mM sodium formate to elute  $\text{InsP}_1$ ; 5x2ml. 0.3M ammonium formate in 0.1M formic acid to elute  $\text{InsP}_2$  and 3x2ml. 0.75M ammonium formate in 0.1M formic acid to elute  $\text{InsP}_3$ .

FIG. 3.8



maintained at approximately this level for up to 30 minutes (Figure 3.9.). After exposure for 60 minutes a slight reduction to 125% of control was seen.

The individual IPs formed by prelabelled astrocyte-enriched cultures in response to 50  $\mu\text{gml}^{-1}$  aqueous PA at 1, 2 (in the absence of lithium), 5 and 30 (in the presence of lithium) minutes are shown in Table 3.5. Increases over control in the  $\text{InsP}_3$  and  $\text{InsP}_2$  fractions were seen after exposure to PA for 1 and 2 minutes, a 74% increase being seen in the  $\text{InsP}_3$  fraction at both time points and the increase in  $\text{InsP}_2$  at 2 minutes (60%) being larger than that at 1 minute (50%). The 1 and 2 minute incubations were carried out in the absence of lithium so no increases were seen in the  $\text{InsP}_1$  fractions. An increase in  $\text{InsP}_3$  accumulation was no longer seen after 5 and 30 minute PA exposure. The  $\text{InsP}_2$  fraction showed an increase over control of 112% at 5 minutes but no increase was detectable after 30 minutes. These incubations were carried out in the presence of lithium and consequently increases were seen in the  $\text{InsP}_1$  fraction of 61% and 84% over control at 5 and 30 minutes respectively.

The individual [ $^3\text{H}$ ]IPs formed by prelabelled astrocytes in response to PA either in solvent or PA aqueous were also examined in the same experiment. Cells were exposed to PA in 0.1%  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) or aqueous PA, both at 100  $\mu\text{gml}^{-1}$ , for 1 minute and compared to the appropriate solvent or aqueous control. Similar increases in the  $\text{InsP}_2$  and  $\text{InsP}_3$  fractions were seen with both types of PA (Table 3.6.)

Figure 3.9. The time course of aqueous PA-induced [<sup>3</sup>H]inositol phosphate accumulation (GPI and IPs).

Astrocyte-enriched cultures (18 div) were prelabelled for 48h and then exposed to PA at 50  $\mu\text{gml}^{-1}$  for the times indicated (in the presence of 5mM lithium choride). Results are mean +/- SEM (n = 3) using 60mm dishes and are expressed as % of control incubations (stopped after lithium preincubation). Mean dpm for control incubations = 1612 dpm. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to control incubation (stopped after lithium preincubation).



FIG. 3.9

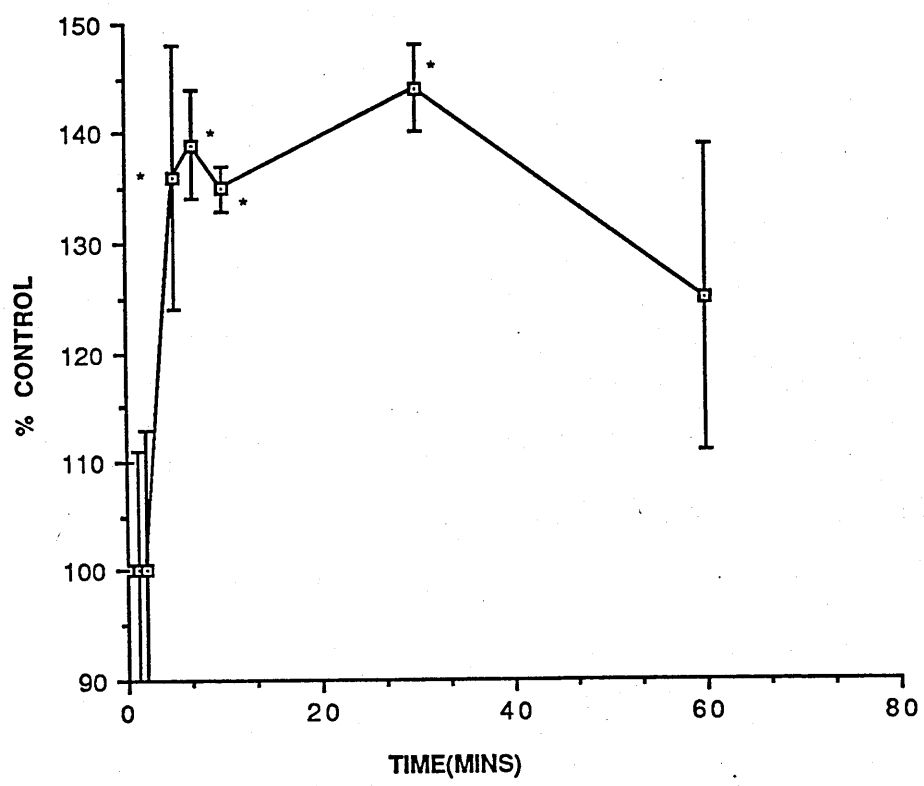


Table 3.5. The time course of PA-induced individual  
[<sup>3</sup>H]inositol phosphate accumulation.

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and then exposed to PA at 50  $\mu\text{gml}^{-1}$  for the times indicated (T = 1 and 2 minutes in the absence of lithium, T = 5 and 30 minutes in the presence of lithium). Results are mean  $\pm$  SEM (n = 3) using 60mm dishes. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each IP fraction compared to appropriate control IP fraction.

Time (mins)	Sample	Total normalised dpm/IP fraction		
		InsP <sub>1</sub>	InsP <sub>2</sub>	InsP <sub>3</sub>
1	Control	4110 $\pm$ 830	5316 $\pm$ 618	1315 $\pm$ 282
	PA	4259 $\pm$ 304	7989 $\pm$ 1043*	2289 $\pm$ 278*
2	Control	3887 $\pm$ 390	5313 $\pm$ 504	1420 $\pm$ 228
	PA	4111 $\pm$ 153	8515 $\pm$ 1548	2474 $\pm$ 117*
5	Control	7222 $\pm$ 1328	4627 $\pm$ 882	950 $\pm$ 214
	PA	11639 $\pm$ 1766*	9820 $\pm$ 1823*	1492 $\pm$ 31
30	Control	7155 $\pm$ 384	7754 $\pm$ 1266	1208 $\pm$ 257
	PA	13151 $\pm$ 3113*	9963 $\pm$ 1360	1107 $\pm$ 279

Table 3.6. The effect of PA in solvent compared to PA aqueous on the accumulation of individual [<sup>3</sup>H]inositol phosphates.

Astrocyte-enriched cultures (18 div) were prelabelled for 72h and then exposed to 100  $\mu\text{gml}^{-1}$  PA in 0.1%  $\text{CHCl}_3$ :MeOH (1:2 v/v) or aqueous PA for 1 minute.

Sample	Total normalised dpm /IP fraction		
	InsP <sub>1</sub>	InsP <sub>2</sub>	InsP <sub>3</sub>
Aqueous control	5804	4078	2557
Aqueous PA	6485	8266	3849
Solvent control	6361	4692	2190
Solvent PA	5533	8282	3069

### 3.3.1.3. Calcium dependence

The calcium requirements of the PA response were determined by exposing prelabelled astrocyte-enriched cultures to 50 or 100  $\mu\text{gml}^{-1}$  PA for 30 minutes under varying extracellular calcium conditions. Incubations were carried out either in the presence of added calcium ( $+\text{Ca}^{++}$ ), in the absence of added calcium ( $-\text{Ca}^{++}$ ) or the absence of added calcium and the presence of EGTA ( $-\text{Ca}^{++} + \text{EGTA}$ ).

PA at 50 or 100  $\mu\text{gml}^{-1}$  in the presence of extracellular calcium elicited a 31% and 56% increase respectively in IPs accumulated when compared to the appropriate control (Table 3.7.). In the absence of added calcium, the calcium concentration being that of the incubation medium, which is approximately 10  $\mu\text{M}$ , PA at 50  $\mu\text{gml}^{-1}$  still elicited an increase in IPs accumulation over control but it was lower than that seen in the presence of added calcium, 12% compared to 31%. PA at either 50 or 100  $\mu\text{gml}^{-1}$  had no effect on IPs accumulation when all available calcium was removed by the presence of EGTA. Calcium dependency was also exhibited by the control incubations.

### 3.3.1.4. Effect of PAs with fatty acids of differing chain lengths.

The effect of PAs of different fatty acid chain length on IPs accumulation was determined by exposing prelabelled astrocyte-enriched cultures to 50  $\mu\text{gml}^{-1}$  PA of differing compositions for 30 minutes. For these experiments lithium

Table 3.7. The calcium dependence of PA-induced [<sup>3</sup>H]inositol phosphate accumulation (GPI and IPs).

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and then exposed to PA at 50 and 100  $\mu\text{gml}^{-1}$  (in the presence of 5mM lithium chloride) for 30 minutes in the presence of 2.5mM calcium chloride, the absence of added calcium or in the absence of added calcium and the presence of 0.5mM EGTA. Results are mean  $\pm$  SEM (n = 3) using 60mm dishes. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to appropriate control.

Sample	Normalised dpm		
	+Ca <sup>++</sup>	-Ca <sup>++</sup>	-Ca <sup>++</sup> + EGTA
Control	1698 $\pm$ 81	885 $\pm$ 14	819 $\pm$ 33
PA (50)	2231 $\pm$ 271 *	989 $\pm$ 25 *	643 $\pm$ 30
Control	1309 $\pm$ 234	ND	372 $\pm$ 59
PA (100)	2038 $\pm$ 257 *	ND	348 $\pm$ 83

ND not determined

was added at the same time as PA. PA from egg yolk lecithin caused an increase in IPs accumulation of 142% of control but none of the other PAs tested, containing lauric (PA di-C<sub>12:0</sub>), palmitic (PA di-C<sub>16:0</sub>) or oleic acid (PA di-C<sub>18:1</sub>), had any effect (Table 3.8.).

The effect of PAs of differing composition on the individual IPs formed was determined by exposing prelabelled astrocyte-enriched cultures to different PAs for 1 minute in the absence of lithium. Only PA from egg yolk lecithin caused significant increases in InsP<sub>3</sub> and InsP<sub>2</sub> accumulation of 151% and 138% of control respectively (Table 3.9.). PA di-C<sub>12:0</sub>, PA containing myristic acid (PA di-C<sub>14:0</sub>) and PA di-C<sub>16:0</sub> had no effect on the accumulation of any IP. However, PA containing C<sub>18:1</sub> did cause a significant increase in InsP<sub>1</sub> and InsP<sub>2</sub> accumulation of 139% and 142% of control respectively.

### 3.3.2. Effect of PA on phosphoinositide labelling of astrocytes.

#### 3.3.2.1. Time Course

The effect of PA on the metabolism of inositol phospholipids was further investigated by studying the recovery of [<sup>3</sup>H]inositol in PtdIns, PtdIns(4)P and PtdIns(4,5)P<sub>2</sub>. Prelabelled astrocyte-enriched cultures were exposed to PA at 50  $\mu\text{gml}^{-1}$  for varying lengths of time. The extracted labelled phospholipids were separated by thin layer chromatography and a representative diagram of the

Table 3.8. The effect of PAs of differing fatty acid chain length on PA-induced [ $^3\text{H}$ ]inositol phosphate accumulation.

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and then exposed to PA di-C<sub>12:0</sub>, PA di-C<sub>16:0</sub>, PA di-C<sub>18:1</sub> and PA egg yolk lecithin at 50  $\mu\text{gml}^{-1}$  for 30 minutes (in the presence of 5mM lithium chloride). Results are mean  $\pm$  SEM (n = 3) using 60mm dishes. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to control (no PA added).

Sample	Normalised dpm	% control
Control	1664 $\pm$ 68	100 $\pm$ 4
PA di-C <sub>12:0</sub>	1362 $\pm$ 150	82 $\pm$ 9
PA di-C <sub>16:0</sub>	1437 $\pm$ 187	86 $\pm$ 11
PA di-C <sub>18:1</sub>	1705 $\pm$ 254	102 $\pm$ 15
PA lecithin	2367 $\pm$ 134	142 $\pm$ 8 *

Table 3.9. The effect of PAs of differing fatty acid chain length on PA-induced individual [ $^3\text{H}$ ]inositol phosphate accumulation.

Astrocyte-enriched cultures (18 div) were prelabelled for 72h and then exposed to PA di-C<sub>12:0</sub>, PA di-C<sub>14:0</sub>, PA di-C<sub>16:0</sub>, PA di-C<sub>18:1</sub> and PA from egg yolk lecithin at 50  $\mu\text{gml}^{-1}$  for 1 minute. Results are mean  $\pm$  SEM (n = 3) using 6 well plates. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to control (no PA added).

Sample	Total dpm/IP fraction		
	InsP <sub>1</sub>	InsP <sub>2</sub>	InsP <sub>3</sub>
Control	4045 $\pm$ 439	3544 $\pm$ 199	933 $\pm$ 90
PA di-C <sub>12:0</sub>	5513 $\pm$ 595	4678 $\pm$ 750	999 $\pm$ 207
PA di-C <sub>14:0</sub>	4738 $\pm$ 197	4209 $\pm$ 454	977 $\pm$ 86
PA di-C <sub>16:0</sub>	5059 $\pm$ 502	4201 $\pm$ 533	1313 $\pm$ 188
PA di-C <sub>18:1</sub>	5628 $\pm$ 618 *	5026 $\pm$ 625 *	1179 $\pm$ 127
PA lecithin	4700 $\pm$ 659	4882 $\pm$ 117 *	1412 $\pm$ 136 *



separation achieved is shown in Figure 3.10. After 1 and 2 minute incubations a reduction of approximately 50% was seen in the amount of radiolabel recovered in PtdIns(4,5)P<sub>2</sub> compared to control incubations (Table 3.10.) and no changes were seen in the labelling of PtdIns or PtdIns(4)P. By 5 minutes an increase in the labelling of both PtdIns(4,5)P<sub>2</sub> and PtdIns(4)P and a decrease in that of PtdIns was found in response to PA.

#### 3.3.2.2. Effect of PAs with fatty acids of differing chain length.

The effect of changing the fatty acid chain length of PA on the inositol phospholipid labelling of prelabelled astrocyte-enriched cultures was investigated by exposing cultures to PA of differing composition for 30 minutes in the presence of lithium. After 30 minutes exposure increases in the % labelling of PtdIns(4,5)P<sub>2</sub> and PtdIns(4)P were seen which seem to be dependent on the presence of longer chain fatty acids (Table 3.11.) which is in agreement with data obtained from IPs accumulation experiments. Chain length dependent decreases were also seen in the labelling of PtdIns.

#### 3.3.3. Effect of PA on <sup>45</sup>Ca<sup>++</sup> efflux from astrocytes.

##### 3.3.3.1. Concentration dependence.

Astrocyte-enriched cultures preloaded with <sup>45</sup>Ca<sup>++</sup> for 24h were exposed to various concentrations of PA. After the

Figure 3.10. A representative diagram of the phosphoinositide separation achieved with the TLC system employing high performance silica gel 60F TLC plates and mobile phase of the following composition:

$\text{CHCl}_3$ :acetone:MeOH:acetic acid: $\text{H}_2\text{O}$   
(40:15:13:12:8 v/v).

Key to samples applied to TLC plate

Sample number	Phospholipid	Source
1	PA	Sigma
2	PtdIns	Sigma
3	PtdIns(4)P	Sigma
4	PtdIns(4,5) $\text{P}_2$	Sigma
5	PA	Serdary
6	Mixture of 1,2,3 and 4	Sigma

Figure 3.10.

SOLVENT  
FRONT

ORIGIN

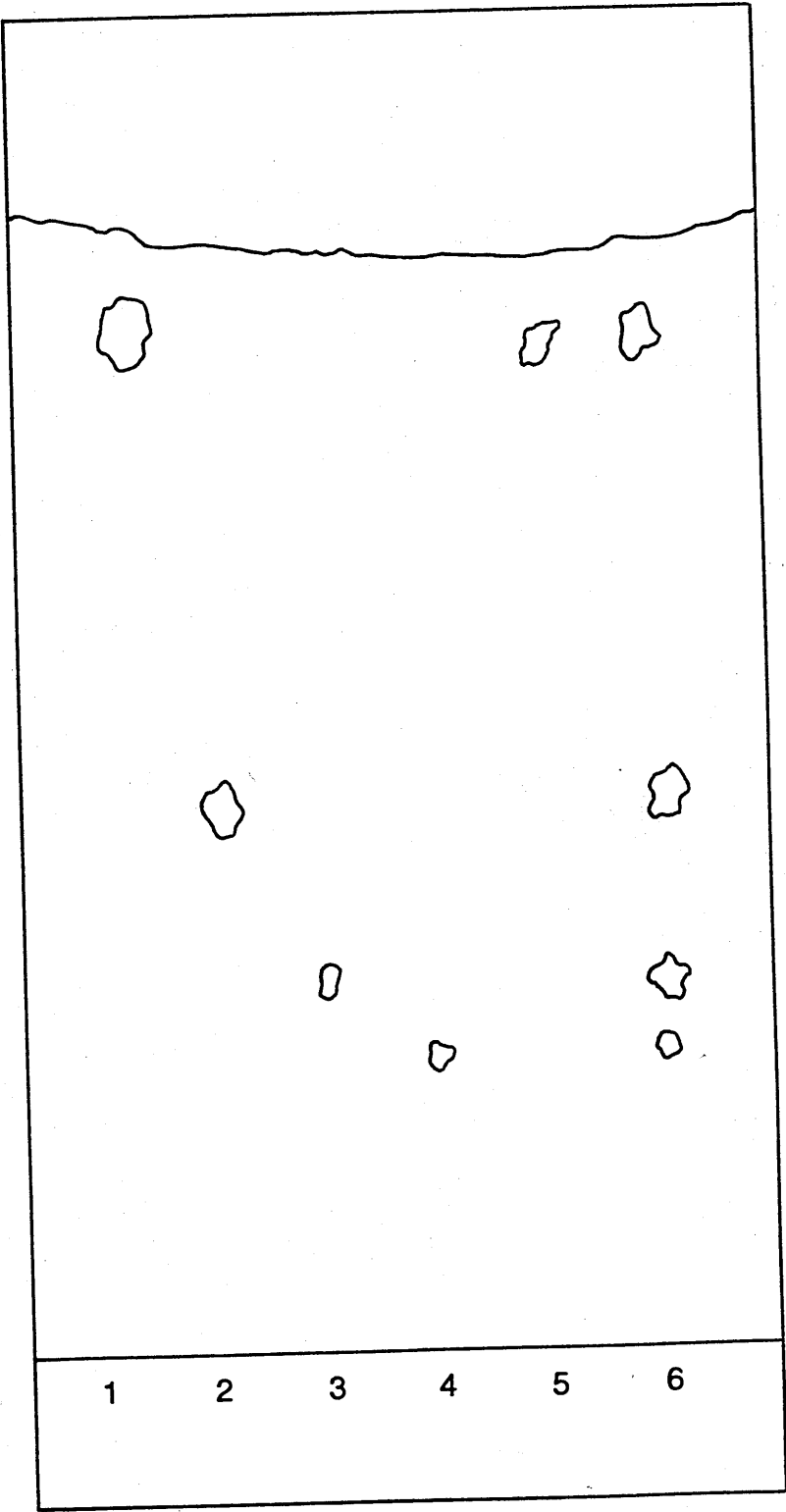


Table 3.10. PA-induced changes in [ $^3\text{H}$ ]inositol phospholipid labelling.

Astrocyte-enriched cultures (18 div) were prelabelled for 24h and then exposed to PA at  $50\mu\text{gml}^{-1}$  for the time indicated. Results are mean  $\pm$  SEM (n) using 60mm dishes and are expressed as % of the total dpm recovered in the phosphoinositides in each phosphoinositide fraction. dpm recovered in each phosphoinositide fraction were as follows: PtdIns 170,000-360,000 dpm; PtdIns(4)P 6,000-10,000 dpm; PtdIns(4,5) $\text{P}_2$  800-1500 dpm. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to control (no PA added).

Time (mins)	Sample	% phosphoinositide labelling		
		PtdIns	PtdIns(4)P	PtdIns(4,5) $\text{P}_2$
1	Control (6)	92.0 $\pm$ 1.2	3.7 $\pm$ 0.6	4.3 $\pm$ 0.9
	PA (7)	93.4 $\pm$ 0.9	4.2 $\pm$ 0.6	2.2 $\pm$ 0.4 *
2	Control (3)	92.9 $\pm$ 1.2	4.0 $\pm$ 0.9	3.1 $\pm$ 0.6
	PA (4)	94.6 $\pm$ 0.4	3.8 $\pm$ 0.5	1.6 $\pm$ 0.4 *
5	Control (1)	95.1	2.3	2.6
	PA (1)	91.9	4.8	3.3

Table 3.11. The effect of PAs of differing fatty acid chain length on [<sup>3</sup>H]inositol phospholipid labelling.

Astrocyte-enriched cultures (18 div) were prelabelled for 72h and then exposed to PAs of different composition at 50  $\mu\text{gml}^{-1}$  for 30 minutes (in the presence of 5mM lithium chloride). Results are from 1 experiment using 60mm dishes. dpm recovered in each phosphoinositide fraction were as follows: Ptd Ins 360,000 dpm; PtdIns(4)P 8,800 dpm; PtdIns(4,5)P<sub>2</sub> 800 dpm.

Sample	% phosphoinositide labelling		
	PtdIns	PtdIns(4)P	PtdIns(4,5)P <sub>2</sub>
Control	96.8	1.8	1.4
PA di-C <sub>12:0</sub>	94.0	3.3	2.7
PA di-C <sub>14:0</sub>	95.3	2.4	2.4
PA di-C <sub>16:0</sub>	96.4	1.8	1.8
PA di-C <sub>18:1</sub>	92.0	4.5	3.4
PA lecithin	83.3	10.1	6.6

basal efflux was established PA from either Lipid Products or Serdary Research Labs, at 1, 10 or 100  $\mu\text{gml}^{-1}$  was added for 1 minute. Both PAs were stored as 100  $\text{mgml}^{-1}$  stocks in  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) and diluted appropriately for use. PA at 100  $\mu\text{gml}^{-1}$  whether from Lipid Products or from Serdary caused an increase in  $^{45}\text{Ca}^{++}$  efflux of 200% and 188% of control respectively (Table 3.12.). No significant effect was seen with 1 or 10  $\mu\text{gml}^{-1}$  PA from either source.

The previous experiment was carried out using a stock solution of PA at 100  $\text{mgml}^{-1}$  in  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v). In view of its effects on PPI turnover the effect of this solvent on  $^{45}\text{Ca}^{++}$  efflux was also investigated. No significant effect of 0.01% or 0.1%  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v) was seen and PA from Serdary at 100  $\mu\text{gml}^{-1}$  in 0.1% v/v solvent still elicited an increase in efflux (Table 3.13.), although the increase was only 20% compared to 88% in the previous experiment.

The effect of aqueous PA on  $^{45}\text{Ca}^{++}$  efflux from astrocytes was also investigated and a significant increase in efflux of 15% over control was seen at 100  $\mu\text{gml}^{-1}$  (Table 3.13.). This increase was similar to that obtained in the experiment outlined above but lower than that obtained in the first experiment described. Again 1 or 10  $\mu\text{gml}^{-1}$  PA were without effect. Thus PA does cause  $^{45}\text{Ca}^{++}$  efflux from astrocytes, but like the stimulation of PPI turnover, the response was found to be variable.

Table 3.12. The effect of two different PAs on  $^{45}\text{Ca}^{++}$  efflux from astrocytes.

Astrocyte-enriched cultures (18 div) preloaded with  $^{45}\text{Ca}^{++}$  were exposed to various concentrations of PA for 1 minute. Results are mean  $\pm$  SEM (n = 3) using 6 well plates and are expressed as % of the efflux sample prior to PA addition. dpm in PA exposure fraction 160-740 dpm. Mean residual dpm in cells = 2894 dpm. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to the efflux sample prior to PA addition.

Sample	% Control	
	Lipid Products PA	Serdary PA
PA 1	105 $\pm$ 1	88 $\pm$ 1
PA 10	116 $\pm$ 9	114 $\pm$ 14
PA 100	200 $\pm$ 27 *	188 $\pm$ 8 *

Table 3.13. PA-induced  $^{45}\text{Ca}^{++}$  efflux from astrocytes.

Astrocyte-enriched cultures (18 div) were preloaded with  $^{45}\text{Ca}^{++}$  for 24h and then exposed to PA at  $100\mu\text{gml}^{-1}$  in 0.1% solvent and compared with the appropriate control, or exposed to aqueous PA at 1, 10 or  $100\mu\text{gml}^{-1}$ . Results are mean  $\pm$  SEM (n) using 6 well plates and are expressed as % of the efflux sample prior to PA addition. dpm in exposure fraction 235-840 dpm. Mean residual dpm in cells = 6926  $\pm$  314. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to the efflux sample prior to PA addition.

Sample	% Control
0.01% solvent	92 $\pm$ 3 (n = 6)
0.1% solvent	96 $\pm$ 6 (n = 4)
PA 100 in 0.1%	120 $\pm$ 6 (n = 6) *
Aqueous PA 1	96 $\pm$ 3 (n = 6)
Aqueous PA 10	109 $\pm$ 8 (n = 6)
Aqueous PA 100	115 $\pm$ 5 (n = 4) *



### 3.3.3.2. Effect of PAs with fatty acids of differing chain length.

The effect of PAs of differing fatty acid chain length on  $^{45}\text{Ca}^{++}$  efflux from astrocytes was investigated by exposing cells to PAs of varying composition for 1 minute. The largest increase in response was seen with PA from egg lecithin, although increases in efflux were seen with PA containing  $\text{C}_{14:0}$ ,  $\text{C}_{16:0}$  and  $\text{C}_{18:1}$  fatty acids (Table 3.14.). The shortest fatty acid chain length PA tested,  $\text{C}_{12:0}$ , had no effect on efflux. There did appear to be a gradation in the  $^{45}\text{Ca}^{++}$  response of astrocytes to differing composition PAs.

### 3.3.4. Effect of PA on [ $^3\text{H}$ ]inositol phosphates accumulation in, and $^{45}\text{Ca}^{++}$ efflux from fibroblast cultures.

The major contaminating cell type of these astrocyte-enriched cultures is fibroblasts of meningeal origin which constitute approximately 5-10 % of the cells present. To assess whether contaminating fibroblasts were responsible for the effect of PA on PPI turnover and the  $^{45}\text{Ca}^{++}$  efflux that was seen, the effect of PA was investigated in confluent fibroblast cultures. The meninges of 5 neonatal rats were used to seed six 6 wells. The mean protein content of these confluent cultures was  $925 \pm 17 \mu\text{g/well}$  ( $n = 12$ ) and the mean DNA content was  $40.7 \pm 2.5 \mu\text{g/well}$  ( $n = 12$ ). Prelabelled fibroblast cultures were exposed to varying concentrations of PA for 30 minutes. PA had no significant effect on IPs accumulation in this cell type

Table 3.14. The effect of PAs of differing composition on  $^{45}\text{Ca}^{++}$  efflux from astrocytes.

Astrocyte-enriched cultures (18 div) were preloaded for 24h with  $^{45}\text{Ca}^{++}$  and then exposed to PAs of differing composition for 1 minute. Results are mean  $\pm$  SEM (n = 9) using 6 well plates from 3 experiments and are expressed as % of the efflux sample prior to PA addition. dpm in PA exposure fraction 150-505 dpm. Mean residual dpm in cells = 2105  $\pm$  129. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to the efflux sample prior to PA addition.

Sample	% control
PA di-C <sub>12:0</sub>	110 $\pm$ 3
PA di-C <sub>14:0</sub>	116 $\pm$ 2 *
PA di-C <sub>16:0</sub>	117 $\pm$ 5 *
PA di-C <sub>18:1</sub>	110 $\pm$ 5 *
PA lecithin	121 $\pm$ 3 *

(Table 3.15).

However  $^{45}\text{Ca}^{++}$  efflux was increased when preloaded astrocytes were exposed to  $100\ \mu\text{gml}^{-1}$  PA (Table 3.15.). The response, a 17% increase over control, was of a similar magnitude to that elicited by PA in astrocytes.

Table 3.15. PA-induced [ $^3\text{H}$ ]inositol phosphate accumulation in and  $^{45}\text{Ca}^{++}$  efflux from fibroblasts.

Fibroblast cultures (19 div) prelabelled with [ $^3\text{H}$ ]myo-inositol for 24h and then exposed to various concentrations of PA (in the presence of 5mM lithium chloride) for 30 minutes. Results are mean  $\pm$  SEM (n = 6) using 6 well plates from 2 experiments. Fibroblast cultures (19 div) preloaded with  $^{45}\text{Ca}^{++}$  for 24h were exposed to varying concentrations of PA for 1 minute. Results are mean  $\pm$  SEM (n = 3) using 6 well plates and are expressed as % of the efflux sample prior to PA addition. dpm in PA exposure fraction 520-685 dpm. Mean residual dpm in cells = 4810  $\pm$  164. ND not determined. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.001 and \*\*\*\* 0.001. Each condition compared to the efflux sample prior to PA addition.

PA conc. ( $\mu\text{gml}^{-1}$ )	IP accumulation Normalised dpm	$^{45}\text{Ca}^{++}$ efflux % control
0	383 $\pm$ 30	
1	426 $\pm$ 26	109 $\pm$ 7
10	465 $\pm$ 34	87 $\pm$ 3
50	440 $\pm$ 27	ND
100	483 $\pm$ 59	117 $\pm$ 7 *

### 3.4. SUMMARY

(i) PA elicited a dose- and time-dependent increase in the accumulation of labelled inositol phosphates and individual inositol phosphates. This stimulation of PPI turnover appeared to be dependent on a low extracellular calcium concentration as well as PA containing longer chain length fatty acids.

(ii) PA was found to cause time-dependent changes in the labelling of inositol phospholipids and this effect of PA was also dependent on longer chain length PAs.

(iii) PA produced  $^{45}\text{Ca}^{++}$  efflux from astrocytes which again was dependent on PA containing longer chain length fatty acids.

(iv) PA had no detectable effect on PPI turnover in fibroblasts but did stimulate  $^{45}\text{Ca}^{++}$  efflux from these cells.

### 3.5. DISCUSSION.

The ability of PA to promote PPI breakdown in astrocytes was demonstrated in this chapter. There does appear to be a correlation between PA-stimulated PPI turnover and PA-induced DNA synthesis in astrocytes. PA had been found to elicit increased [ $^3\text{H}$ ]thymidine incorporation into astrocyte DNA and increase the DNA content of astrocyte cultures (Chapter 2) over a similar concentration range to that which had been found to stimulate astrocyte PPI turnover. Agreement was also seen between the data obtained here for the effect of PA on PPI breakdown and data obtained for the mitogenic action of PA on astroglial cultures from previous work by Murphy and Pearce (1988a).

In other studies on Rat-1 and NIH 3T3 fibroblasts PA stimulated PPI turnover and increased [ $^3\text{H}$ ]thymidine incorporation over the same concentration range (Moolenaar et al 1986; Yu et al 1988). Siegmann (1987) found that PA either in the form of liposomes or in buffer also increased [ $^3\text{H}$ ]thymidine incorporation into 3T3 cells. Although the concentration of PA used in this study was higher and the exposure time only 30 minutes, the cells may still have been exposed to a lower level of PA for a longer period of time due to the presence of some residual PA after the cells had been washed.

PA caused a dose- and time-dependent increase in total IPs accumulated but to determine whether PA was activating PL C and stimulating  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis the individual IPs

formed had to be investigated. The changes that were seen in the formation of the individual IPs are in general agreement with those that are associated with PA-induced changes in other cell types. The pattern of accumulation of individual IPs in astrocytes is similar to those seen by other workers using fibroblasts exposed to PA. Using A431 cells Moolenaar et al (1986) found increased  $\text{InsP}_3$  accumulation after 30 seconds exposure to PA at  $50 \mu\text{gml}^{-1}$ . Murayama and Ui (1987a) showed that PA caused the accumulation of  $\text{InsP}_3$  in 3T3 cells which preceded that of  $\text{InsP}_2$ , which in turn was seen before an increase in  $\text{InsP}_1$ . The increases that were seen in  $\text{InsP}_1$  and  $\text{InsP}_2$  may also have been due to the action of a calcium-dependent PL C on  $\text{PtdIns}$  and  $\text{PtdIns}(4)\text{P}$  (Majerus et al 1985). Not all the  $\text{InsP}_1$  and  $\text{InsP}_2$  seen could have been produced from  $\text{InsP}_3$  since the amount of  $\text{PtdIns}(4)\text{P}$  and  $\text{PtdIns}(4,5)\text{P}_2$  present in the cell membrane is much smaller than  $\text{PtdIns}$ . Between 92% and 95% of the  $[^3\text{H}]$ inositol radiolabel incorporated into astrocyte cell membranes is associated with  $\text{PtdIns}$  and the remaining  $[^3\text{H}]$ inositol radiolabel is equally distributed in  $\text{PtdIns}(4)\text{P}$  and  $\text{PtdIns}(4,5)\text{P}_2$ . Calcium mobilisation could thus have initiated the hydrolysis of  $\text{PtdIns}$  or  $\text{PtdIns}(4)\text{P}$  by a calcium-dependent PL C to liberate  $\text{InsP}_1$  and  $\text{InsP}_2$ .

These changes in individual IPs are also in agreement with the effect of other calcium mobilising stimuli that activate PPI turnover in their target cells. Thrombin activation of fibroblasts leads to  $\text{InsP}_3$  and  $\text{InsP}_2$  formation which precedes that of  $\text{InsP}_1$  (L'Allemain et al 1986) and stimulation by PDGF elicits  $\text{InsP}_3$  and  $\text{InsP}_2$  formation before

that of  $\text{InsP}_1$  (Berridge et al 1984). Similar patterns of accumulation of individual IPs have also been seen in astrocytes in response to agonists whose receptors are known to be linked to PPI turnover. After short periods of exposure, up to 1 minute, increases are seen in  $\text{InsP}_3$  and  $\text{InsP}_2$  accumulation in response to carbachol and noradrenaline but after longer exposure, for example up to 60 minutes, increases in  $\text{InsP}_1$  and  $\text{InsP}_2$  only are seen (Pearce et al 1986a). Glutamate and ATP have also been shown to bring about early changes in the accumulation of  $\text{InsP}_3$  and  $\text{InsP}_2$  in astrocytes (Pearce et al 1986b; 1989).

The changes seen in the labelling of the inositol phospholipids of astrocytes in response to PA are also in agreement with the initial action of PA being that of promoting the hydrolysis of  $\text{PtdIns}(4)\text{P}$  by PL C. The effect of PA on inositol phospholipid metabolism has not been investigated in similar studies looking at the effect of PA on PPI turnover in other cell types, but the action of PA on astrocytes agrees with that seen in response to agonists that are known to mobilise calcium in their target cell by activating PPI breakdown. The action of acetylcholine on iris smooth muscle initially produced a loss of  $^{32}\text{P}$  radiolabel from labelled  $\text{PtdIns}(4,5)\text{P}_2$  (Abdel-Latif 1986) and the action of bradykinin on NG 108-15 cells, a neuroblastoma glioma hybrid, also caused a loss of  $^{32}\text{P}$  from  $\text{PtdIns}(4,5)\text{P}_2$  (Yano et al 1984). At later time points however the stimulation of fibroblasts with PDGF causes a loss of  $[^3\text{H}]$ inositol label from  $\text{PtdIns}$  and an increase in label in  $\text{PtdIns}(4)\text{P}$  and  $\text{PtdIns}(4,5)\text{P}_2$  (Berridge et al 1984),



as PtdIns is used to replenish the PtdIns(4)P and PtdIns(4,5)P<sub>2</sub> lipid pools.

PA was shown to have an effect on calcium mobilisation in astrocytes as demonstrated by increased efflux of <sup>45</sup>Ca<sup>++</sup> from prelabelled astrocytes. This effect of PA on astrocytes is in agreement with its action on other cell types. PA at similar concentrations to those that were used in this study caused a transient increase in [Ca<sup>++</sup>]<sub>i</sub> in A431 cells (Moolenaar et al 1986) and osteoblasts (Kawase and Suzuki 1988) as well as eliciting Ca<sup>++</sup> efflux from perfused rat liver (Altin and Bygrave 1987). Increased <sup>45</sup>Ca<sup>++</sup> efflux has been reported in glial cells in response to a number of agonists whose action involves the promotion of PPI turnover. Carbachol, glutamate and noradrenaline elicit <sup>45</sup>Ca<sup>++</sup> efflux from 138 MG glioma cells (Lazarewicz and Kanje 1981) and ATP, carbachol, glutamate and noradrenaline promote <sup>45</sup>Ca<sup>++</sup> efflux from cerebral astrocytes (Pearce and Murphy 1988; Pearce et al 1986b; 1989).

The omission of calcium reduced the total IPs accumulated in response to PA in astrocytes and the inclusion of EGTA abolished the response to PA altogether in this study which is in contrast to the results obtained using 3T3 cells where the omission of calcium from the incubation medium had no effect on the action of PA (Murayama and Ui 1987a).

Moolenaar et al (1986) using A431 cells found that the addition of EGTA caused only a slight reduction in the effect that PA had on increasing [Ca<sup>++</sup>]<sub>i</sub> and the action of PA on increasing [Ca<sup>++</sup>]<sub>i</sub> in osteoblasts was also independent

of extracellular calcium (Kawase and Suzuki 1988). This lack of dependence on extracellular calcium for the action of PA has also been demonstrated in human platelet membranes where the addition of EGTA had no effect on the stimulatory effect of PA on  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis by PL C (Jackowski and Rock 1989). The action of PA on astrocytes appears to be different from that seen in other cell types since it does demonstrate a requirement for micromolar concentrations of calcium. The actions of carbachol and noradrenaline on astrocytes are little changed when calcium is omitted from the incubation medium (Pearce et al 1986a), but a reduction in response was seen when astrocytes were exposed to carbachol, noradrenaline or glutamate in calcium-free incubation medium containing EGTA (Pearce et al 1986a; 1986b). PA's calcium dependence seems to be different from that of conventional agonists linked to PPI turnover in astrocytes. Could these differences be explained by PA acting as a calcium ionophore?

From work done with the calcium ionophore A23187 on astrocytes (Pearce et al 1987) it does not seem that PA is acting like an ionophore in these cells since different patterns of accumulation of individual IPs and different changes in the labelling of inositol phospholipids are seen. A23187 stimulates PPI turnover in astrocytes, but increases were seen in  $\text{InsP}_2$  and  $\text{InsP}_1$  and unlike PA, no increase in  $\text{InsP}_3$  was seen. Changes were also seen in the labelling of  $\text{PtdIns}$  and  $\text{PtdIns}(4)\text{P}$  in astrocytes in response to A23187, but no change was seen in  $\text{PtdIns}(4,5)\text{P}_2$  labelling whereas change was seen in response to PA.

A number of mechanisms have however been suggested for the way in which PA may be stimulating PL C hydrolysis of PtdIns(4,5)P<sub>2</sub>. PA could be released from the cell to interact with specific PA binding sites that are linked to PPI turnover (Murayama and Ui 1987a) or PA could cause membrane perturbation that would make PtdIns(4,5)P<sub>2</sub> more susceptible to PL C attack (Moolenaar et al 1986).

PA had no effect on the total IPs accumulation in fibroblasts of meningeal origin under the same conditions that promoted PPI turnover in astrocytes. The PPI turnover seen in astrocytes in response to PA was therefore not due to the presence of contaminating fibroblasts. PA did however stimulate PPI breakdown in 3T3 (Murayama and Ui 1987a) and A431 fibroblasts (Moolenaar et al 1986). Murayama and Ui (1987a) demonstrated an effect of PA in 3T3 cells but not in platelets. These differences between different cell types were explained by Murayama and Ui (1987a) as being due to the presence or absence of specific binding sites for PA.

Further evidence for the possible existence of specific binding sites for PA comes from studies using other cells that show that PA of a particular structural conformation appears to be required for this phospholipid to have its maximal effect. This requirement for the presence of long chain fatty acids in PA is seen in astrocytes both for its effect on PPI turnover and on <sup>45</sup>Ca<sup>++</sup> efflux. The effect of PA in 3T3 cells (Murayama and Ui 1987a) was also dependent on the presence of long chain unsaturated fatty acids. These workers observed that PA containing the saturated fatty acid

lauric acid ( $C_{12:0}$ ) was able to antagonise the inhibitory action of PA containing the unsaturated fatty acid oleic acid ( $C_{18:1}$ ). Similar structural requirements of PA were demonstrated by Moolenaar et al (1986). The addition of PA from lecithin (the naturally occurring form of PA) to A431 cells elicited a transient increase in  $[Ca^{++}]_i$  but the response to PA containing palmitic acid ( $C_{16:0}$ ) was variable, and that to PAs containing myristic acid ( $C_{14:0}$ ) or lauric acid ( $C_{12:0}$ ) had no effect. Skaper et al (1989) found that only PA from lecithin was capable of inhibiting the morphological changes associated with increased levels of cAMP in astroglial cells, PA containing oleic acid ( $C_{18:1}$ ), stearic acid ( $C_{18:0}$ ), palmitic acid ( $C_{16:0}$ ), myristic acid ( $C_{14:0}$ ) or lauric acid ( $C_{12:0}$ ) were without effect. Shortening the chain length of the fatty acids in PA also reduced the effectiveness of PA in activating the hydrolysis of  $PtdIns(4,5)P_2$  by PL C in human platelet membranes (Jackowski and Rock 1989). The effect of PA on inositol phospholipid labelling in astrocytes was also dependent on the presence of long chain fatty acids for maximal effect.

Further support for the existence of specific PA binding sites comes from studies that show that the action of PA in a number of different cell systems seems to be specific for PA since other phospholipids or free fatty acids cannot mimick its effects. PS, PC and arachidonic acid (AA) were unable to mimick the effects of PA on 3T3 cells (Murayama and Ui 1987a).  $PtdIns$ , PC and the fatty acids AA,  $C_{18:1}$  and  $C_{18:0}$  were unable to increase  $[Ca^{++}]_i$  in A431 cells

(Moolenaar et al 1986). PE, PC, PtdIns and PS had no effect on the activation of PtdIns(4,5)P<sub>2</sub> hydrolysis by PL C (Jackowski and Rock 1989) or the conversion of epithelial-like astrocytes to process-bearing cells induced by increased cAMP levels (Skaper et al 1989). PC, PE and PtdIns had no effect on [Ca<sup>++</sup>]<sub>i</sub> in osteoblasts (Kawase and Suzuki 1988).

Evidence for the mode of action of PA being that of perturbing the membrane structure has come from work showing the effectiveness of endogenously-produced PA. The action of PL D, which cleaves membrane phospholipids to yield PA, has been shown to elevate [Ca<sup>++</sup>]<sub>i</sub> in A431 cells (Moolenaar et al 1986) and to inhibit the changes in astrocyte cell morphology (Skaper et al 1989). PA produced within the cell, during PPI turnover, could be acting at the membrane to amplify the initial signal that activated PPI turnover.

The question remains, however, as to how PA affects PPI turnover and DNA synthesis in astrocytes? Is PA released from astrocytes to interact with specific cell surface receptors or is PA produced at the cell membrane to amplify the original signal that stimulated PPI breakdown? The following chapter details investigations into how PA might be working in astrocytes by studying the production of labelled PA in astrocytes and whether or not PA is released. The effect that blocking PA production has on PPI turnover and calcium mobilisation is also detailed.

## CHAPTER 4 IS PHOSPHATIDIC ACID RELEASED FROM ASTROCYTES FOLLOWING PPI TURNOVER?

### 4.1. INTRODUCTION

PA has been shown to have a growth factor-like action on astrocytes (Chapter 2), and to activate PPI turnover and mobilise calcium in astrocytes (Chapter 3). The possibility that endogenously formed PA may be able to act in a similar manner to exogenous PA has led to the proposal that PA may be involved in a positive feedback loop (Moolenaar et al 1986). PA produced within the cell during PPI turnover could by acting on PL C to amplify the initial calcium-mobilising stimulus.

The mechanisms which have been proposed for the way in which PA could itself be activating the very system that leads to its production can be thought of as falling into two categories. The first category involves the direct or indirect interaction of PA with PL C and the second group are membrane effects which PA may be having that lead to the activation of PPI metabolism.

Evidence in support of an interaction between PA and PL C has come from work on 3T3 fibroblasts by Murayama and Ui (1987). Changes in the fatty acid composition of PA led to dramatic differences in the effect of PA in these cells and its effect was specific for PA and was not mimicked by other phospholipids. These points led these workers to propose the existence of specific PA binding sites on the

extracellular surface of 3T3 cells. Rabbit platelets were found to be unresponsive to PA in this same study and this was attributed to the lack of such PA receptors on these cells. The interaction of PA with membrane-bound receptors could be a means by which PA brings about its effect since compounds which contain long chain fatty acids are too hydrophobic to insert into the membrane (Takai et al 1985). Jackowski and Rock (1989) have also suggested a direct activation of human platelet membrane PL C by PA.

Murayama and Ui (1987) also demonstrated that platelets released PA in response to thrombin, an agonist which is known to activate PPI breakdown. This increase in radioactivity associated with PA outside the cell led to the proposal that PA formed during PPI turnover is released from the cell to interact with receptors on the cell surface.

The other way in which PA may be having its effect is by its ability to change the cell membrane lipid bilayer structure. The lipid configuration (Irvine et al 1984a) and substrate presentation (Irvine et al 1985) in the membrane have been found to be important factors in controlling the activity of rat brain phosphodiesterase on  $\text{PtdIns}(4,5)\text{P}_2$ . The activity of phosphodiesterase has been shown to be dependent on its phospholipid environment. Choline-containing phospholipids were found to have an inhibitory action on PL C while PA and PS were stimulatory (Irvine et al 1979a). Anionic phospholipids, such as PA, could effect the physical form of  $\text{PtdIns}(4,5)\text{P}_2$  making it more susceptible to phospholipase attack (Jackowski and Rock 1989). The fatty acid content of

membranes also has a role to play in controlling PPI turnover (Irvine et al 1979b). Membrane perturbation from an organised tightly packed bilayer to an undefined structure has been reported to increase the hydrolysis of bilayer forming phospholipids by phospholipases (Dawson et al 1983). Unsaturated long chain fatty acid diacylglycerols were found to be more effective than those containing saturated short chain fatty acids at increasing phospholipase activity (Dawson et al 1984). PA containing palmitic ( $C_{16:0}$ ) or stearic acid ( $C_{18:0}$ ) fatty acids were more effective than that containing decanoic acid ( $C_{10:0}$ ) because they had a greater disruptive effect on the packing of phospholipids (Dawson et al 1980).

So the main question about how PA may be activating PPI turnover is whether it is produced and then released from the cell to interact with cell surface receptors or if once produced within the cell it has local effects on the cell membrane?

PA production during PPI turnover is by the action of the enzyme DG kinase on DG (Lapetina and Cuatrecasas 1979). DG kinase catalyses the phosphorylation of DG to PA using ATP and enables DG to be re-used in the  $\text{PtdIns}(4,5)\text{P}_2$  cycle (Lin et al 1986). It is an efficient mechanism for terminating the action of the intracellular second messenger DG and controlling the concentration of DG. DG kinase has been reported to be present in both the soluble and membrane-bound fractions from pig brain (Kanoh et al 1989), the cytosol from bovine brain as well as in a number of other



animal tissues (Lin et al 1986). A membrane-bound DG kinase has been isolated that specifically phosphorylates DG containing arachidonic acid (AA) (MacDonald et al 1988).

In order to assess the contribution of intracellularly formed PA in amplifying a calcium-mobilising stimulus a way of inhibiting its production is required. De Chaffoy de Courcelles et al (1985) found that the compound 6-[2-[4-[(4-fluorophenyl) phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5 H-thiazolo-[3,2-a] pyrimidin-5-one (R59 022) inhibited the  $^{32}\text{P}$  labelling of PA and increased the levels of DG in response to thrombin in platelets and red blood cell membranes. R59 022 was found to be a specific inhibitor of DG kinase, it had no effect on PtdIns or PtdIns(4)P kinase. R59 022 was also reported to be a weak dopamine  $\text{D}_2$ ,  $\alpha_1$ -adrenergic, and histamine  $\text{H}_1$  antagonist and a strong serotonin  $5\text{HT}_2$  antagonist (de Chaffoy de Courcelles 1985). R59 022 increased the levels of DG in neutrophils in response to agonists which act via PL C (Muid et al 1987). An increase in DG-stimulated PKC activity was observed when R59 022 was added to fMet-Leu-Phe stimulated neutrophils and to thrombin-stimulated platelets (Muid et al 1987; Halenda and Rehm 1987).

Consequently there are a number of questions to be answered about the action of PA on astrocytes. Is PA formation seen in astrocytes in response to agonists linked to PPI turnover? Is this PA being released from astrocytes to then interact with cell surface receptors or is it having its effects within the cell at the membrane? Inositol

phospholipids have been reported to be enriched in arachidonic acid (AA) compared to other phospholipids (Holub and Kuksis 1978; Dudley and Spector 1986). By labelling astrocyte cultures with [ $^3\text{H}$ ]AA the production and release of labelled PA from labelled phospholipids in response to various agonists could be investigated.

If PA is involved in amplifying the initial calcium-mobilising stimulus in astrocytes, blocking its production by the presence of the DG kinase inhibitor R59 022 should have an effect on PPI turnover and  $\text{Ca}^{++}$  mobilisation in response to agonists linked to PPI turnover in these cells. The effect of R59 022 on carbachol-stimulated PA production and IPs formation in astrocytes, and  $^{45}\text{Ca}^{++}$  efflux from astrocytes was therefore investigated.

In the remainder of this chapter I shall present evidence to show that:

(i) Carbachol, noradrenaline and ATP increased the formation of labelled PA at the cell membrane but had no effect on the release of PA from astrocytes into the extracellular medium.

(ii) R59 022 inhibited astrocyte PA production and  $\text{Ca}^{++}$  mobilisation but had no effect on IPs formation.

## 4.2. METHODS AND MATERIALS

### 4.2.1. Astrocyte cultures

Astrocyte-enriched cultures were prepared as described in Chapter 2, section 2.2.1..

### 4.2.2. Labelling of astrocyte phospholipids

#### 4.2.2.1. Incubations

Cells grown for 18-22 div in 6 well plates were labelled in GM containing  $0.25 \mu\text{Ci ml}^{-1}$  [ $^3\text{H}$ ]AA. After rinsing twice with PSS, incubations were carried out in fresh PSS for 1 minute with the appropriate agonist additions. The incubation medium was removed and, after washing twice with PSS, the cells were harvested. Lipids were extracted from both the incubation medium and the cells using 1ml  $\text{CHCl}_3:\text{MeOH}$  (1:2 v/v). 0.3ml  $\text{CHCl}_3$  and 0.3ml  $\text{H}_2\text{O}$  were added and after thorough mixing the aqueous and organic phases were separated by centrifugation for 10 minutes at 180g at  $4^\circ\text{C}$ . Aliquots of the lipid and aqueous fractions of the incubation medium and the cells were taken for liquid scintillation counting.

#### 4.2.2.2. Thin layer chromatography

The phospholipid composition of the lipid fraction of the incubation medium and cell samples was determined by thin layer chromatography according to the method of Tolbert et

al (1980). Each sample was dried and spiked with a phospholipids standards mixture in  $\text{CHCl}_3$  containing  $10\mu\text{g}$  of each of the following phospholipids: PA, PtdIns, PS, PC and PE. Separation was performed on 20cm x 20cm silica gel 60F TLC plates by a mobile phase containing  $\text{CHCl}_3:\text{MeOH}:28\% \text{NH}_4\text{OH}:\text{H}_2\text{O}$  (65:35:3:2 v/v). After drying at room temperature, the plates were exposed to iodine vapour to visualise the phospholipids. The following  $R_f$  values were obtained for the phospholipid standards: PA 0.09; PtdIns 0.17; PS 0.19; PC 0.29 and PE 0.54. The spots were scraped into 0.5ml  $\text{H}_2\text{O}$  and 8ml Emulsifier Safe scintillant added. The  $[\text{}^3\text{H}]\text{AA}$  content of the phospholipids was then determined by liquid scintillation counting.

#### 4.2.3. DG kinase inhibitor (R59 022) studies

A 50mM stock solution of R59 022 in ethanol was freshly prepared. This was diluted with 5mM HCl and then with  $\text{H}_2\text{O}$  according to the method of de Chaffoy de Courcelles et al (1985) to give a final R59 022 concentration of  $10\mu\text{M}$  in each incubation. The cells were well washed to ensure that all growth medium was removed so that R59 022 was not absorbed by any plasma proteins that may have been present. R59 022 or the vehicle alone was added to the appropriate incubations. The effect of R59 022 on carbachol-stimulated PA production, PPI metabolism and  $^{45}\text{Ca}^{++}$  efflux was investigated as outlined below.

#### 4.2.3.1. PA production

PA production in [ $^3\text{H}$ ]AA-labelled astrocytes in response to carbachol was determined as previously described in section 4.2.2. R59 022 or vehicle was added 5 minutes prior to carbachol.

#### 4.2.3.2. Phosphoinositide metabolism.

Cells grown for 18-22 div in 6 well plates were labelled for 24h in GM containing  $1\ \mu\text{Ci ml}^{-1}$  [ $^3\text{H}$ ]myo-inositol. The labelling medium was removed, the cells rinsed twice with PSS and incubated for 45 minutes in inositol-free PSS. The cells were again rinsed and pre-incubated for 5 minutes in fresh PSS containing R59 022 or vehicle. The appropriate carbachol addition was then made and the cells incubated for 1 minute. The incubation was stopped by removing the incubation medium, rinsing twice with PSS and then adding 1ml MeOH to each well (Pearce et al 1989). The cells from all 6 wells from each 6 well plate were combined and extracted with 3ml  $\text{CHCl}_3$  and 3ml deionised  $\text{H}_2\text{O}$ . After mixing, the suspensions were centrifuged at 180g for 10 minutes at  $4^\circ\text{C}$  to separate the aqueous and organic phases. An aliquot of the  $\text{CHCl}_3$  layer was removed for liquid scintillation counting. An aliquot of the aqueous layer was diluted with  $\text{H}_2\text{O}$  and the individual IPs were separated by anion exchange chromatography as described earlier in Chapter 3, section 3.2.2.3.

#### 4.2.3.3. Measurement of $^{45}\text{Ca}^{++}$ efflux.

$^{45}\text{Ca}^{++}$  efflux from pre-labelled astrocytes grown for 18-22 div in 6 well plates in response to various concentrations of carbachol was determined as described in Chapter 3, section 3.2.3. R59 022 or vehicle was added at the same time as the appropriate concentration of carbachol.

#### 4.2.4. Statistical analysis

Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01, \*\*\*\* 0.005.

#### 4.2.5. Materials

The following phospholipid standards: the sodium salt of PtdIns from bovine brain and PS, PC and PE from bovine brain and iodine were obtained from Sigma.  $\text{NH}_3$  solution was obtained from BDH. PA was obtained from Serdary Research Labs., Canada. TLC plates were obtained from Merck. R59 022 was obtained from Janssen Life Science Products, Wantage, UK. [5,6,8,9,11,12,14,15- $^3\text{H}$ ]arachidonic acid in ethanol (210Ci/mmol), myo-[2- $^3\text{H}$ ]inositol in water:ethanol 9:1 v/v (15Ci/mmol) and  $^{45}\text{Ca}$  calcium chloride in 0.15M HCl (15mCi/mg calcium) were obtained from Amersham International PLC.

### 4.3. RESULTS

#### 4.3.1. [<sup>3</sup>H]arachidonic acid labelling of astrocyte membrane lipids.

The time course of [<sup>3</sup>H]AA labelling of astrocyte membrane lipids was investigated to determine the optimum labelling time for this series of experiments. Astrocyte-enriched cultures grown in 6 well plates were exposed to labelled GM for 1, 3, 20 or 24 hours. The labelling medium and the cells were extracted and the radioactivity present in the lipid and aqueous phase at each time point was determined by liquid scintillation counting. An increase in the incorporation of [<sup>3</sup>H]AA into cellular lipid was seen from 7% of the label applied at 1h to 44% at 24h (Figure 4.1a). A small increase was also seen in the radiolabel associated with the aqueous fraction of the cells but this only accounted for from 0.5% to 1.5% of the label applied over the same time period. The [<sup>3</sup>H]AA content of the labelling medium fell from 40% of the radiolabel applied at 1h to 17% and 18% at 20h and 24h respectively (Figure 4.1b). An increase in the radioactivity associated with the aqueous fraction of the labelling medium from 25% to 32% was also seen. A labelling time of 24h was chosen for further experiments because this gave the largest incorporation of [<sup>3</sup>H]AA into the lipids of these cultures.

[<sup>3</sup>H]AA is incorporated into a number of different lipid classes, for example, mono-, di- and tri-glycerides as well as phospholipids. The breakdown of these labelled lipids



Figure 4.1. The time course of [ $^3\text{H}$ ]AA labelling of membrane lipids of astrocytes.

Astrocyte-enriched cultures were exposed to labelled GM for the times indicated. The radioactivity associated with the lipid and aqueous phase of the cells (a) and the labelling GM (b) was determined. Results are mean dpm  $\pm$  SEM (n = 3 wells from a 6 well plate).

FIG. 4.1 A

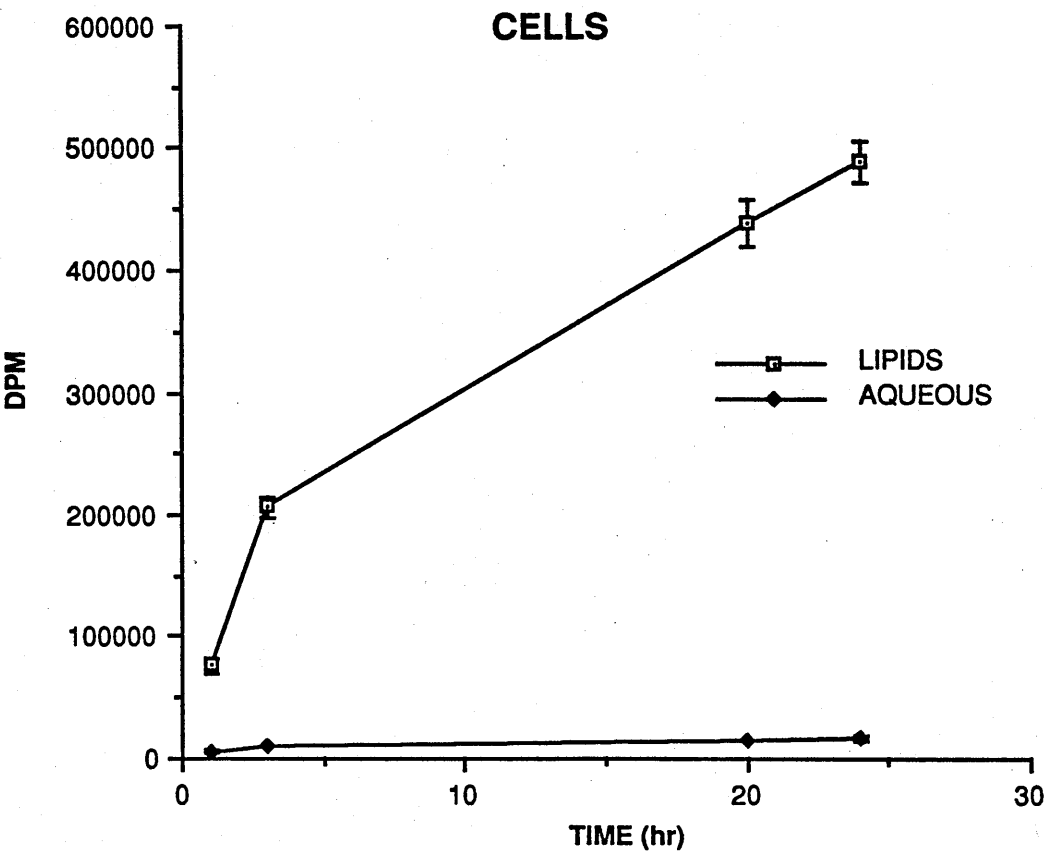
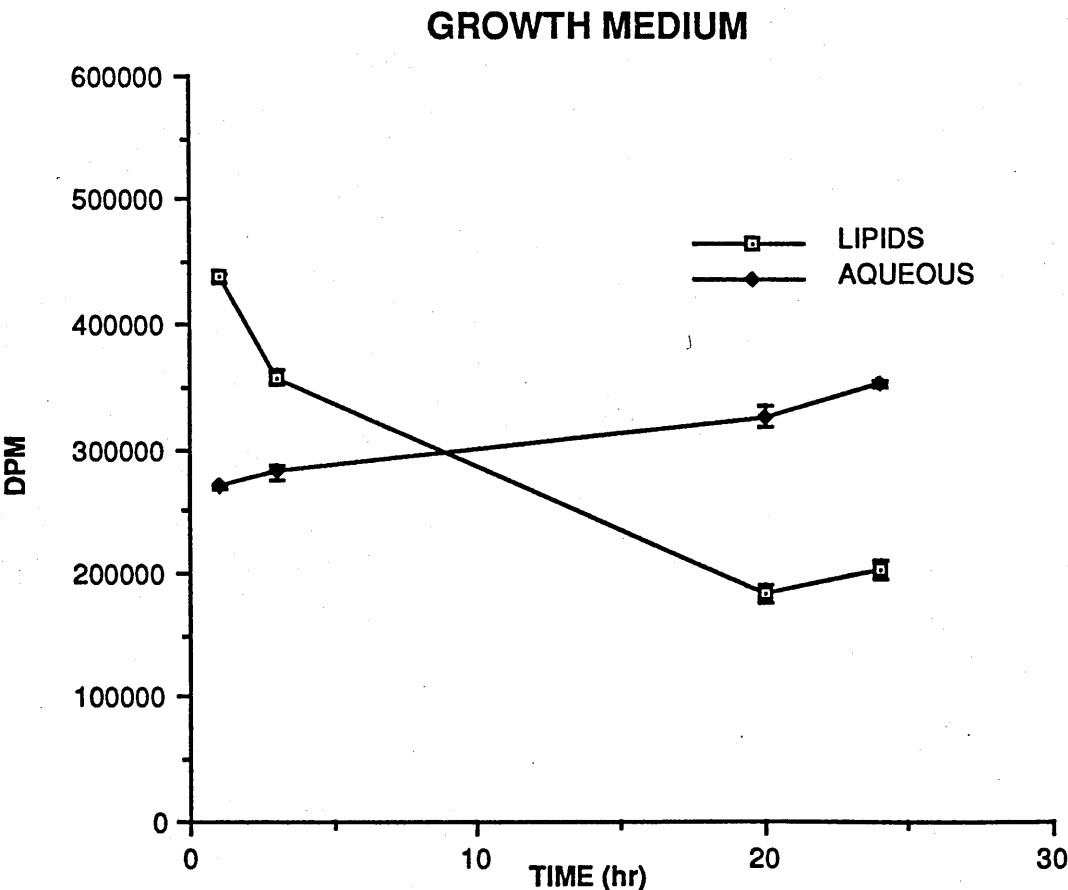


FIG. 4.1 B



and the incorporation of radiolabel into aqueous products can be seen from Fig. 4.1., although the counts involved are very small compared to those incorporated into lipids. The TLC system employed in this study only determined the incorporation of [ $^3\text{H}$ ]AA into phospholipids and does not visualise incorporation into other lipids or radiolabelled aqueous products. This may explain the variation in recovery of radiolabel that is seen in this set of experiments.

4.3.2. The effect of carbachol, noradrenaline and ATP on PA production at the cell membrane of astrocytes.

Cultures were prelabelled with [ $^3\text{H}$ ]AA and then exposed to carbachol (1mM), noradrenaline (1  $\mu\text{M}$ ) or ATP (100  $\mu\text{M}$ ) for 1 minute to determine whether PA was produced at the cell membrane. After incubation the cells were rinsed and then extracted. The phospholipid composition of an aliquot of the lipid fraction was determined by TLC. A representative diagram of the phospholipid separation achieved with this TLC system is shown in Figure 4.2. The dpm recovered in the PA fraction is expressed as a % of the total dpm recovered in the four phospholipid fractions, PA, PtdIns/PS, PC and PE. A one minute incubation with carbachol, noradrenaline or ATP caused an increase in radiolabel associated with PA compared to control (Figure 4.3). Carbachol elicited a 118% of control increase in radiolabel associated with PA, and both noradrenaline and ATP caused a 129% of control increase.

Figure 4.2. A representative diagram of the phospholipid separation achieved with the TLC system employing silica gel 60F TLC plates and mobile phase of the following composition:

$\text{CHCl}_3:\text{MeOH}:28\% \text{NH}_4\text{OH}:\text{H}_2\text{O}$  (130:70:6:4 v/v).

Key to samples applied to TLC plate

Sample number	Phospholipid	Source
1	Sample solvent	
2	PA	Sigma
3	PA	Lipid Products
4	PA	Serdary
5	PtdIns	Sigma
6	PS	Sigma
7	PC	Sigma
8	PE	Sigma
9	Sample solvent	

Figure 4.2.

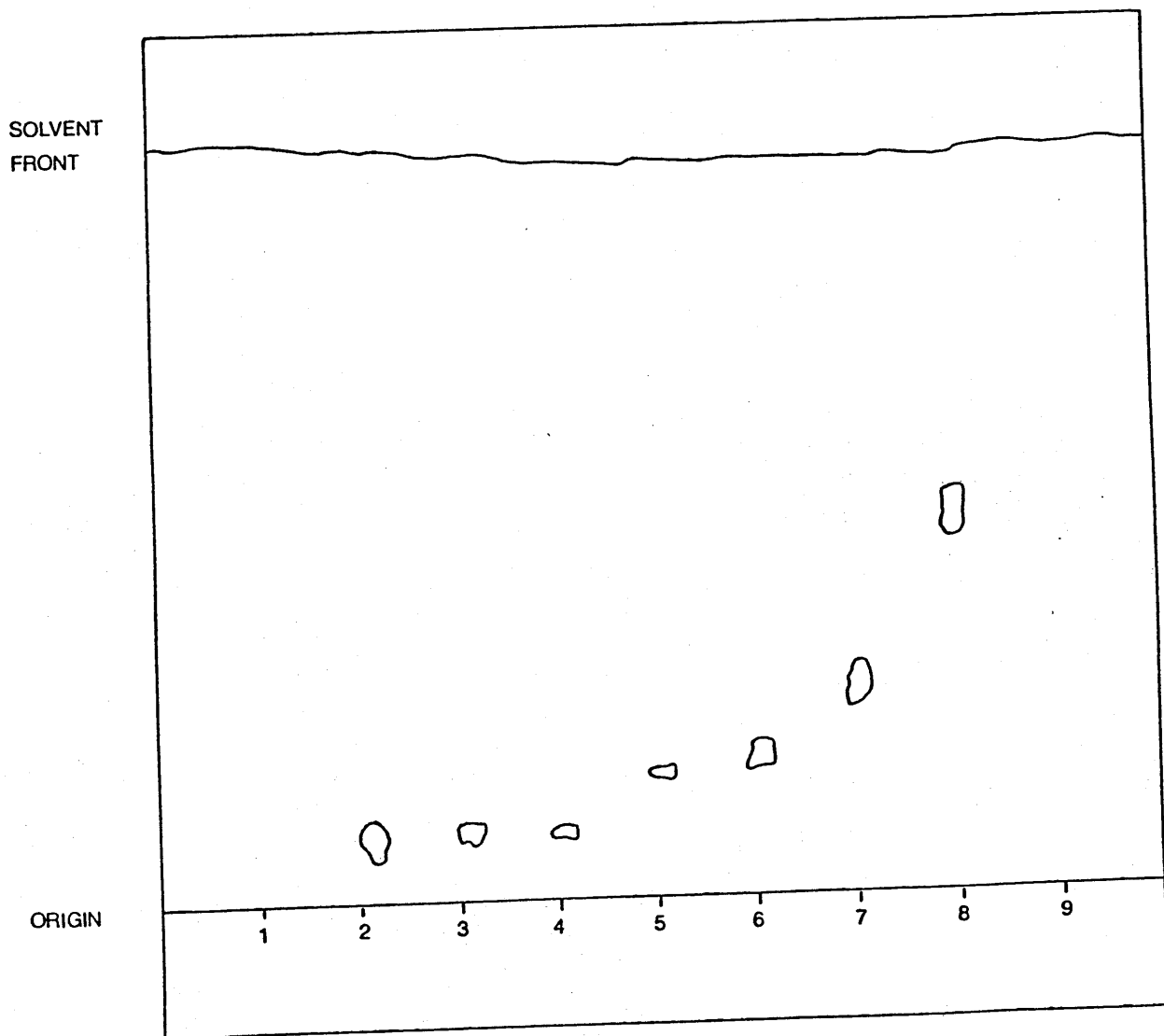
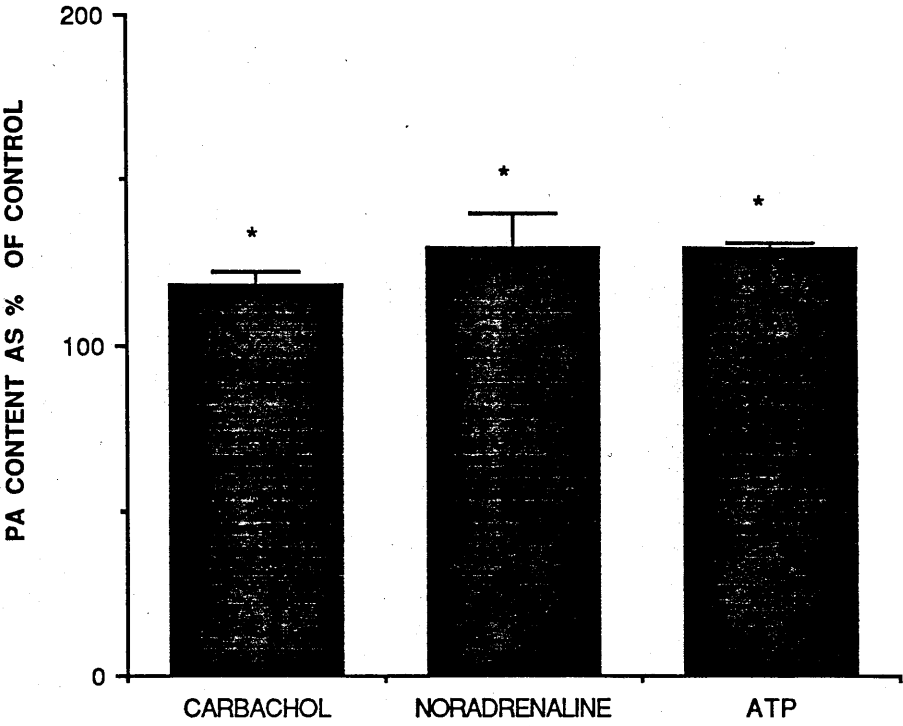


Figure 4.3. The effect of agonists that activate PPI turnover on PA production at the cell membrane of astrocytes.

[<sup>3</sup>H]AA-labelled astrocytes were exposed to the appropriate agonist for 1 minute, the cells were then extracted and the phospholipid composition analysed by TLC. The PA content is expressed as % of control incubations (no agonist addition). Results are mean +/- SEM (n = 3 wells from a 6 well plate). Total dpm recovered in the four phospholipid fractions for control incubations = 68802, 59854, and 62116. The counts recovered from the TLC plate in the four phospholipid fractions expressed as a % of the total counts applied to the plate, the mean recovery, was 27%. dpm associated with PA in control incubations = 1117, 1155 and 1204. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to control incubations (no agonist added).

FIG. 4.3



4.3.3. The effect of carbachol, noradrenaline and ATP on PA release from astrocytes.

[<sup>3</sup>H]AA-labelled cultures were exposed to the agonists for 1 minute as in the previous experiment. Release of PA into the extracellular medium was investigated by combining the incubation medium from three samples for extraction. An aliquot of the lipid fraction was then analysed by TLC. As can be seen from Table 4.1 very low counts were seen in the lipid fraction of the incubation medium, and when analysed by TLC the amount of radioactivity recovered in the four different phospholipid fractions was not appreciably greater than background.

No PA release was seen from astrocytes in response to agonists that are known to stimulate PPI turnover in these cells, even though PA production at the cell membrane was seen in response to these agonists under the same conditions.

4.3.4. The effect of DG kinase inhibitor, R59 022, on PA production at the cell membrane of astrocytes.

[<sup>3</sup>H]AA-labelled cultures were exposed to carbachol at 0.1 and 1mM for 1 minute in the absence and presence of the DG kinase inhibitor R59 022. The cells were extracted and an aliquot of the lipid fraction analysed by TLC. Carbachol at 0.1 and 1mM caused increases of 30% and 15% respectively in the % of the total dpm recovered that was associated with PA when compared to control (Figure 4.4a and b). The presence



Table 4.1. The radioactivity associated with the lipid fraction extracted from the combined incubation medium (IM) samples of agonist-stimulated astrocytes.

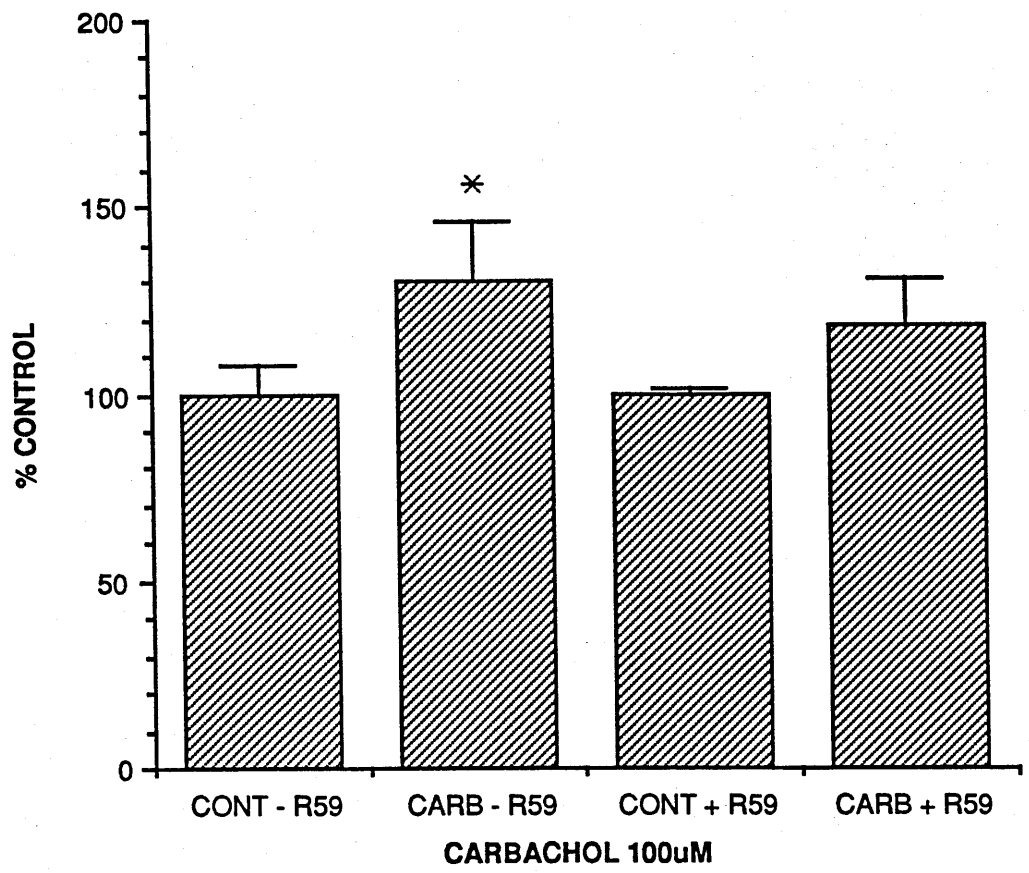
Sample	IM Lipids		dpm from TLC		
	dpm	PA	PI/PS	PC	PE
Control	2268	72	86	151	107
	2268	74	84	103	82
	2304	66	64	101	90
Noradrenaline (1 $\mu$ M)	2628	60	58	98	59
	2232	63	65	116	75
	2340	62	65	106	60
Carbachol (1mM)	2412	66	56	101	83
	2736	69	64	93	69
	2448	60	71	101	69
ATP (100 $\mu$ M)	2592	68	88	124	72
	2412	64	72	114	70
	3096	70	63	100	64

Background samples from top and bottom of plates were 120, 95, 36 and 35 dpm.

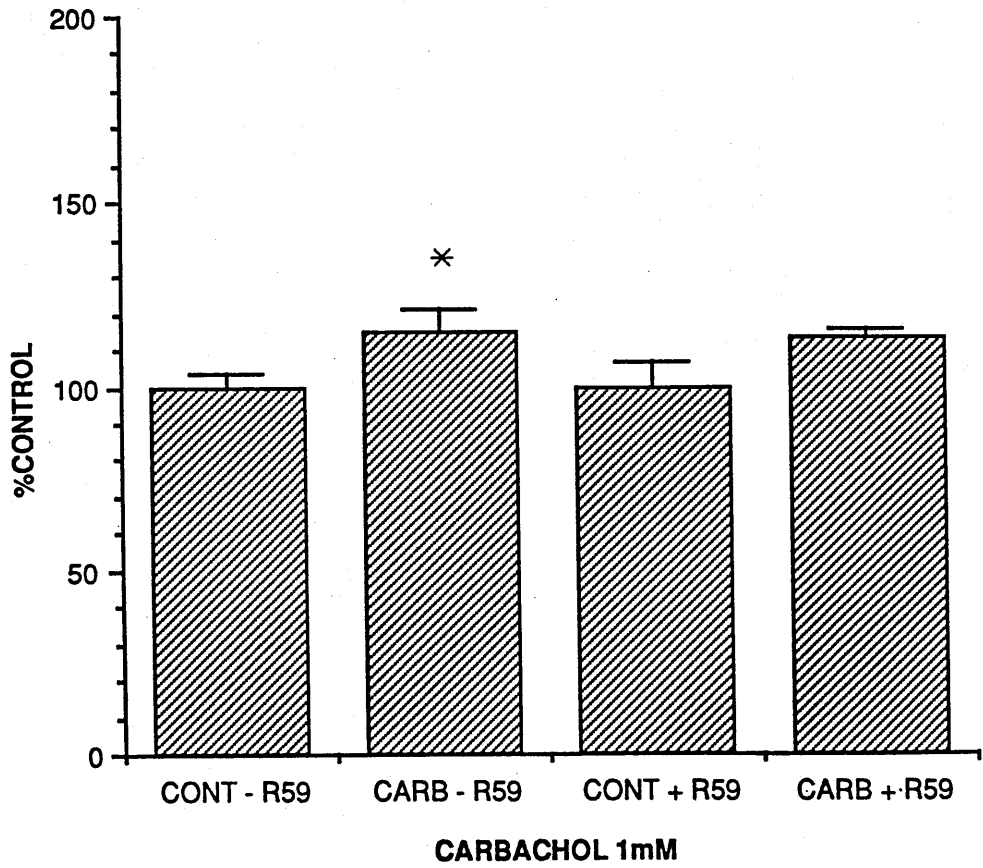
Figure 4.4. The effect of DG kinase inhibitor, R59 022, on carbachol-stimulated PA production.

[<sup>3</sup>H]AA-labelled astrocytes (18-22 div) were exposed to carbachol at 0.1mM (a) or 1mM (b) for 1 minute in the absence or presence of 10  $\mu$ M R59 022. PA content is expressed as % of the appropriate control incubation. Results are mean  $\pm$  SEM (n = 3 wells from a 6 well plate). Total dpm recovered in (a) for control-R59 022 incubations = 114589, 129385 and 103451, and for control+R59 022 incubations = 126456, 121097 and 124830. The counts recovered from the TLC plate in the four phospholipid fractions expressed as a % of the total counts applied to the plate, the mean recovery, was 42%. dpm associated with PA in (a) for control-R59 022 incubations = 1521, 1481 and 1587 and for control+R59 022 incubations = 1522, 1476 and 1547. Total dpm recovered in (b) for control-R59 022 incubations = 63301, 62296 and 89421, and for control+R59 022 incubations = 76074, 73341 and 77336. Mean recovery was 20%. dpm associated with PA in (b) for control-R59 022 incubations = 656, 636 and 822 and for control+R59 022 incubations = 954, 716 and 815. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to appropriate control incubation (no carbachol added).

**FIG. 4.4 A**



**FIG. 4.4 B**



of 10  $\mu$ M R59 022 inhibited PA formation in response to carbachol at 0.1 and 1mM.

4.3.5. The effect of DG kinase inhibitor, R59 022, on carbachol-induced phosphoinositide metabolism.

[ $^3$ H]inositol prelabelled astrocytes were exposed to carbachol at 0.1 and 1mM for 1 minute in the absence or presence of 10  $\mu$ M R59 022. The individual IPs formed in response to carbachol were determined. Increases were seen in the individual IPs formed in response to carbachol whether R59 022 was present or not (Table 4.2).

4.3.6. The effect of DG kinase inhibitor, R59 022, on carbachol-induced  $^{45}\text{Ca}^{++}$  efflux.

Astrocytes prelabelled with  $^{45}\text{Ca}^{++}$  were exposed to various concentrations of carbachol for 1 minute in the absence or presence of R59 022. Increases in  $^{45}\text{Ca}^{++}$  efflux from astrocytes were seen in response to carbachol at 1, 10, 100 and 1000  $\mu$ M. The addition of 10  $\mu$ M R59 022 reduced the effect that 1000  $\mu$ M carbachol had on  $^{45}\text{Ca}^{++}$  efflux. The efflux in response to 1, 10 and 100  $\mu$ M carbachol was unaffected by the presence of R59 022 (Table 4.3.).

Table 4.2. The effect of DG kinase inhibitor, R59 022, on carbachol-stimulated PPI turnover.

[<sup>3</sup>H]inositol prelabelled astrocytes (18-22 div) were exposed to carbachol at 0.1 or 1mM for 1 minute in the absence or presence of 10  $\mu$ M R59 022. Results are expressed as % of appropriate control incubations and are mean +/- SEM (n = 6 using a 6 well plate from 2 experiments). Mean normalised dpm/IP fraction for carbachol at 0.1mM incubations were as follows: InsP<sub>1</sub> = 5177 dpm, InsP<sub>2</sub> = 7101 dpm and InsP<sub>3</sub> = 2383 dpm for control-R59 022 incubations and InsP<sub>1</sub> = 4977 dpm, InsP<sub>2</sub> = 6369 dpm and InsP<sub>3</sub> = 2163 dpm for control+R59 022 incubations. Mean normalised dpm/IP fraction for carbachol at 1mM incubations were as follows: InsP<sub>1</sub> = 4704 dpm, InsP<sub>2</sub> = 5266 dpm and InsP<sub>3</sub> = 1147 dpm for control-R59 022 incubations and InsP<sub>1</sub> = 4396 dpm, InsP<sub>2</sub> = 4754 dpm and InsP<sub>3</sub> = 1234 dpm for control+R59 022 incubations. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each condition compared to appropriate control incubation (no carbachol added).

		% control		
[Carb]	+/- R59 022	InsP <sub>1</sub>	InsP <sub>2</sub>	InsP <sub>3</sub>
0.1mM	-R59 022	116+/- 4***	114+/- 6	123+/-11
	+R59 022	118+/- 6****	125+/- 6***	133+/-11**
1mM	-R59 022	134+/- 8***	108+/- 9	121+/- 4***
	+R59 022	130+/- 5***	134+/- 7***	129+/- 5***

Table 4.3. The effect of DG kinase inhibitor, R59 022, on carbachol-stimulated  $^{45}\text{Ca}^{++}$  efflux.

Astrocytes preloaded with  $^{45}\text{Ca}^{++}$  were exposed to 1, 10, 100 or 1000  $\mu\text{M}$  carbachol for 1 minute in the absence or presence of 10  $\mu\text{M}$  R59 022. The results are expressed as the % of the efflux sample prior to carbachol addition (control sample) and are mean  $\pm$  SEM (n = 6 wells from a 6 well plate from 3 experiments). Mean dpm in carbachol exposure fraction was 134, 122, 136 and 195 dpm for 1, 10, 100 and 1000  $\mu\text{M}$  carbachol. Mean residual dpm in cells = 476 dpm. Statistical analysis was carried out using the Mann-Whitney U test. Various degrees of statistical significance are indicated as follows:- \* p level 0.05, \*\* 0.025, \*\*\* 0.01 and \*\*\*\* 0.001. Each +R59 022 incubation compared to appropriate -R59 022 incubation.

[Carbachol] $\mu\text{M}$	% control	
	- R59 022	+ R59 022
1	144 $\pm$ 9	130 $\pm$ 13
10	163 $\pm$ 13	153 $\pm$ 8
100	181 $\pm$ 16	175 $\pm$ 10
1000	201 $\pm$ 11	165 $\pm$ 9 *

#### 4.4. SUMMARY

(i) Carbachol, ATP and noradrenaline caused an increase in the formation of labelled PA at the cell membrane of astrocytes but these agonists did not evoke PA release from these cells under the same conditions.

(ii) R59 022 inhibited carbachol-stimulated PA production at the astrocyte cell membrane and  $^{45}\text{Ca}^{++}$  efflux from astrocytes but had no effect on carbachol-induced IPs formation.

#### 4.5. DISCUSSION

PA formation in astrocytes was found in response to carbachol, noradrenaline and ATP, (this chapter) agonists that are known to be linked to PPI turnover in these cells (Pearce et al 1986; Pearce et al 1989). PA formation was assumed to be from the breakdown of inositol phospholipids to yield DG, which is then phosphorylated by DG kinase to give PA (Lapetina and Cuatrecasas 1979; Lapetina 1982). Enhanced incorporation of radiolabel into PA and PtdIns has been seen in a number of other tissues which showed agonist-stimulated PtdIns breakdown (Michell 1975; Berridge 1984; Fisher and Agranoff 1985; Hokin 1985). It is now known that the initial reaction involved in phosphoinositide metabolism is PtdIns(4,5)P<sub>2</sub> hydrolysis. A decrease in the labelling of PtdIns(4,5)P<sub>2</sub> and an increase in that of PtdIns and PA is seen (Abdel-Latif 1986). PPI turnover has now been reported in all tissues in which PtdIns turnover had previously been investigated. PPI turnover has been seen in nervous tissue including rat brain and guinea pig synaptosomes (Abdel-Latif 1986).

PA formation has been seen in other astroglial cells in response to agonists that activate inositol phospholipid turnover. Acetylcholine stimulation of [<sup>14</sup>C]AA prelabelled C62B glioma cells led to an early increase in the amount of radiolabel associated with PA (De George et al 1986a), and the stimulation of rat astroglia cells by acetylcholine, noradrenaline, glutamate or histamine also led to an increase in the accumulation of PA (De George et al 1986b).



Although PA formation was seen in astrocytes in response to carbachol, noradrenaline and ATP, no release of PA from astrocytes was seen in response to these same agonists under the same conditions. PA release from [<sup>3</sup>H]AA-labelled thrombin-stimulated platelets into the extracellular medium was seen by Murayama and Ui (1987a), but in this same study no PA release was seen from 3T3 cells. These cell differences were used to support the hypothesis of the existence of specific PA receptors.

The release of PA from the cell to interact with a specific cell surface receptor for PA may however not be necessary for PA to have its effect. PA can be transported across the plasma membrane to interact with PL C by a pathway of lipid uptake involving lipid dephosphorylation and rephosphorylation steps and transbilayer movement. PA has been shown to insert into the outer leaflet of the plasma membrane of Chinese hamster fibroblasts (Pagano et al 1981). Here it is dephosphorylated to DG, which is rapidly translocated to the cytoplasmic face of the plasma membrane and then phosphorylated to PA (Pagano and Longmuir 1985).

If PA is not released but can still interact with membrane-bound PL C perhaps this is occurring by localised membrane perturbation. DG can increase the activity of liver phospholipase A<sub>1</sub>, intestinal mucosal phospholipase A<sub>2</sub> and human platelet PtdIns phosphodiesterase on bilayer-forming phospholipids, for example, PC and PtdIns, by perturbing the tightly-packed bilayer structure (Dawson et al 1983). This effect of DG depends on the presence of long chain,

unsaturated fatty acids for maximum activity (Dawson et al 1984). DG and molecules of analogous structure, for example PA, could have a dramatic effect on cell membrane enzyme behaviour. The phospholipid environment of rat brain PtdIns phosphodiesterase has also been shown to be very important. PC was found to have an inhibitory effect on the hydrolysis of PtdIns whereas PA and PS were stimulatory (Irvine et al 1979a). These anionic phospholipids can act as activators by changing the physical form of the substrate in these assay systems and making the substrate more susceptible to enzymic attack (Dawson et al 1980). The lipid bilayer configuration and the substrate presentation have also been found to be important in controlling the activity of the phosphodiesterase from rat brain on PtdIns(4,5)P<sub>2</sub> (Irvine et al 1984a; 1985). Certain unsaturated fatty acids, for example oleic acid (C<sub>18:1</sub> cis), have been found to stimulate PPI turnover in human platelets and human umbilical vein endothelial cells (Miles et al 1988; 1989). In contrast, the saturated fatty acid stearic acid (C<sub>18:0</sub>) was found to have only a small effect and the trans isomer elaidic acid (C<sub>18:1</sub> trans) was without effect. Exogenous fatty acids readily intercalate into the membrane and produce significant changes in the packing of lipid molecules. Cis-unsaturated fatty acids are taken up into more fluid membrane domains where membrane proteins are located. Unsaturated fatty acids may exert their effect by increasing membrane fluidity in the region of these membrane proteins. Trans-unsaturated or saturated fatty acids have little effect on membrane fluidity. The production of PA in the astrocyte cell membrane in response to agonists that

stimulate PPI turnover may provide a local amplification system to sustain agonist-induced PPI turnover (Dawson et al 1980). PA may be acting at the astrocyte cell membrane by affecting the membrane fluidity in the region of PL C.

If PA production is involved in the maintenance of agonist-induced PPI turnover the inhibition of its production should have an effect on inositol phosphate formation and calcium mobilisation. The specific DG kinase inhibitor R59 022 inhibits PA formation in platelets in response to thrombin, which activates PPI turnover (De Chaffoy de Courcelles et al 1985; Nunn and Watson 1987). Inhibition of PA formation in astrocytes in response to carbachol was seen in the presence of this compound.

Inhibition of PA production reduced the effect of carbachol on  $^{45}\text{Ca}^{++}$  efflux but had no effect on carbachol-induced inositol phosphate formation in astrocytes. This is in contrast to the results obtained by Nunn and Watson (1987) using R59 022 in thrombin-stimulated human platelets. The formation of inositol phosphates and the mobilisation of calcium was not affected by R59 022 in response to thrombin. In this present study and in that carried out by Nunn and Watson (1987) a simple anion exchange chromatography method was used for the separation of the individual inositol phosphates, which does not allow for the identification of the individual  $\text{InsP}_3$  isomers,  $\text{Ins}(1,3,4)\text{P}_3$  and  $\text{Ins}(1,4,5)\text{P}_3$ . In the presence of the DG kinase inhibitor a reduction in the calcium mobilising isomer,  $\text{Ins}(1,4,5)\text{P}_3$ , may have occurred that was not detectable in this or the previous

study thus accounting for the effect of R59 022 on carbachol-induced calcium mobilisation. The lack of effect of R59 022 on thrombin-induced calcium mobilisation may reflect differences in the extent that different cell types use PA production to amplify agonist-induced PPI turnover.

## CHAPTER 5 GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The main aim of this thesis was to extend previous findings on the mitogenic effect of PA on astrocytes and to determine whether PA-stimulated PPI turnover could be responsible for this effect.

Chapter 2 showed that primary cultures of neonatal rat cerebral cortical astrocytes provide a means of investigating the proliferative action of a number of mitogens. Astroglial proliferation was seen in response to insulin, PDGF and PA as measured by [<sup>3</sup>H]thymidine incorporation into DNA. Further investigations into the mitogenic action of PA using a DNA content assay showed that its effect was dependent upon a particular structural configuration for maximal effect.

In this study investigation of the proliferative response of cerebral cortex astrocytes was undertaken. Extensive work has not yet been performed on the effect of various growth factors, whether their action be linked to protein tyrosine kinase activation or increased PPI metabolism, on astrocytes from different CNS regions. However, PDGF, a growth factor whose action is thought to involve the stimulation of PPI turnover (Habenicht et al 1981), has been found to stimulate proliferation of rat cerebral astrocytes in this study and that of Besnard et al (1987) but to have no effect on astrocytes obtained from rat cerebellum (Kniss and Burry 1988). These findings suggest that regional differences may exist in the response of astrocytes to growth factors whose

action involves stimulated PPI turnover. Whether differences are also seen in the response to PA of astrocytes from different brain regions could be investigated.

PA-stimulated PPI turnover in astrocytes was determined by the accumulation of labelled total inositol phosphates and individual inositol phosphates, as well as changes in the labelling of inositol phospholipids (Chapter 3). Increased  $^{45}\text{Ca}^{++}$  efflux from astrocytes, a measure of intracellular calcium mobilisation, was also seen in response to PA. As was demonstrated for the mitogenic action of PA in Chapter 2, all these effects were dependent on the presence of PA containing long chain fatty acids suggesting a specificity for PA of a similar structure to that of the endogenous molecule. This stimulation of PPI turnover by PA in astrocytes was not due to the presence of fibroblasts in these cultures.

Regional differences have been seen in the PPI turnover response of cerebral, cerebellar and spinal cord astrocytes to a number of neuropeptides (Cholewinski et al 1988). Astrocytes from all three regions were found to respond to bradykinin, eledoisin and neurokinin  $\beta$  and cerebral and cerebellar astroglial cells responded to oxytocin and vasopressin. Only cerebral astrocytes responded to adrenocorticotrophic hormone, and neurokinin  $\alpha$  and substance P elicited PPI turnover in spinal cord astrocytes only. Differences have also been seen in  $\beta$ -adrenoreceptors, GFAP expression, glutamine synthetase activity and glutamate uptake in astrocyte cultures from different brain regions

(Hansson 1988). These properties were also affected by the age of the animal at the time of astrocyte culture and the age of astrocytes used. Evidence from the study of Cholewinski et al (1988) mentioned above showed that bradykinin elicited the same PPI turnover response in astrocytes from the cerebral cortex, the cerebellum and the spinal cord regardless of the age of astrocytes in culture. These regional differences in astroglial properties could reflect astrocyte specialisations dependent upon varying neuronal requirements. Obviously it would be worth determining whether PA elicited different PPI turnover responses in astrocytes from different CNS regions.

A direct link between PA-stimulated PPI turnover and PA-induced cell division in astrocytes has not been established in this study but a correlation between PA's mitogenic stimulation of astrocytes and PA-induced increased PPI hydrolysis in these cells was demonstrated. A causal link between PA-stimulated mitogenesis and PPI turnover could be established by investigating the effect of activating the separate branches of the PPI turnover signal transduction pathway. Activation of PKC was found to be involved in the mitogenic action of PA on astrocytes since PKC depletion by prolonged exposure to phorbol ester, a direct activator of PKC (Castagna et al 1982), abolished this effect of PA (Chapter 2). Another way of investigating the role of PKC activation in PA-induced astroglial proliferation would be to use the DG kinase inhibitor, R59 022, to increase the levels of DG, the endogenous activator of PKC. In Swiss 3T3 cells potentiation of PDGF- and bombesin-induced DNA

synthesis and increased PKC activity has been demonstrated in the presence of R59 022 (Morris et al 1988). Further evidence for a role for PKC activation in astroglial proliferation comes from work by Murphy et al (1987) and Bhat (1989) which showed that phorbol ester was mitogenic for astrocytes. Short term activation of PKC with phorbol ester results in inhibition of agonist-induced PPI turnover and calcium mobilisation (Berridge 1987b). Investigation of the effect of this negative feedback mechanism on PA-induced mitogenesis could provide further evidence of a link between stimulated PPI turnover and mitogenesis in response to PA. Unfortunately, direct activation of the calcium mobilising branch of the PPI breakdown signal transduction pathway by employing ionophores has disadvantages in mitogenesis experiments. The long incubation times which are used have a detrimental effect on the cells under investigation, for example, A23187 has been found to be toxic for astrocytes after 48h incubation (Fawthrop and Evans 1987; Murphy et al unpubl.).

The direct involvement of PPI hydrolysis in PA-induced mitogenesis could also be demonstrated by inhibition of PL C, the enzyme responsible for  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis. Neomycin has been proposed to inhibit PL C (Cockcroft and Gomperts 1985) and it has been found to inhibit thrombin-induced mitogenesis and  $\text{Ins}(1,4,5)\text{P}_3$  production in platelets (Carney et al 1985). The specificity of neomycin however remains to be established.

A direct requirement of  $\text{PtdIns}(4,5)\text{P}_2$  hydrolysis for the



action of PDGF and bombesin on NIH 3T3 cells has recently been demonstrated by the use of a specific antibody to  $\text{PtdIns}(4,5)\text{P}_2$  which inhibited PDGF- and bombesin-induced mitogenesis (Matuoka et al 1988). The necessity of  $\text{PtdIns}(4,5)\text{P}_2$  breakdown for PA-induced mitogenesis could be elucidated using similar antibody studies.

The first evidence for a physical association between a component of the PPI breakdown pathway and activated protein tyrosine kinases has recently emerged. A  $\text{PtdIns}$  kinase that phosphorylates the 3' hydroxyl of  $\text{PtdIns}$  to yield  $\text{PtdIns}(3)\text{P}$  has been identified (Whitman et al 1988). Novel 3' phosphorylated phosphoinositides may be involved in a separate signalling pathway used exclusively by mitogenic agents. The action of PA on 3' phosphorylated phosphoinositides could also provide evidence of a link between PA-induced mitogenesis and PPI turnover.

The possibility that endogenous PA, formed during receptor-stimulated PPI breakdown could act in a similar manner to exogenously applied PA, to amplify the initial calcium mobilising stimulus was investigated in Chapter 4. PA was found to be produced at the cell membrane in response to agonists known to be linked to PPI turnover in astrocytes, but was not released from the cell and therefore must have its effect locally at the membrane. The inhibition of PA production by the DG kinase inhibitor, R59 022, inhibited carbachol-induced intracellular calcium mobilisation but had no effect on PPI turnover in response to carbachol.

A role for PA in amplifying extracellular calcium mobilising signals was first suggested by Dawson et al (1980) and interest in PA as an intracellular messenger has recently been renewed in the light of PA's growth factor-like action in a number of fibroblast cell types (Moolenaar et al 1986; Siegmann 1987; Yu et al 1988). Preliminary investigations into the way in which PA may play a role in a local amplification system suggested that PA produced at the cell membrane interacted with PL C to sustain receptor-activated PPI turnover (Chapter 4) or that it may act by altering the membrane in such a way as to make  $\text{PtdIns}(4,5)\text{P}_2$  more susceptible to attack by PL C. Much more information is required before a model of how PA actually produces its effect at the cell membrane can be postulated.

Further investigations of the basic mechanism of action of PA on PPI hydrolysis could involve the determination of whether G proteins are involved. G proteins have been found to couple surface receptors with PL C in a number of different cells (Cockcroft and Gomperts 1985; Gonzales and Crews 1985; Hepler and Harden 1986). A G protein has been implicated in the coupling of astrocyte muscarinic cholinergic receptors in astrocytes to PL C. Using an astrocyte membrane preparation an enhancement of the PPI hydrolysis elicited by a stable analogue of GTP was seen in the presence of carbachol (Pearce et al 1988). The involvement of a G protein in PA's action on PPI turnover could be determined by investigating if GTP was required for the stimulation of PPI metabolism by PA in astrocyte membrane preparations and whether PA can enhance the PPI

hydrolysis elicited by stable analogues of GTP or  $\text{AlF}_4^-$ , which are known to stimulate PL C (Taylor and Merritt 1986).

Phorbol ester-induced down regulation of astroglial muscarinic cholinergic and  $\alpha_1$ -adrenergic receptors linked to PPI breakdown has been reported (Pearce et al 1988). Short term exposure to phorbol esters inhibits agonist-induced PPI turnover. Could PA-induced PPI hydrolysis also be regulated in a similar manner?

Evidence for a role for PA in intracellular signalling has also come from agonist-induced PC turnover. Signal transduction may also occur via PC cycles (for review see Pelech and Vance 1989). PC may be hydrolysed in response to a number of stimuli in a number of cell types to yield metabolites that had generally been accepted to be products of phosphoinositide breakdown. Changes in the levels of PA precede those of DG in vasopressin-stimulated rat hepatocytes as well as in f-methionyl-leucyl-phenylalanine-stimulated neutrophils (Bocckino et al 1987; Agwu et al 1989). These workers proposed that PA was not formed by PL C action on phosphoinositides but by the action of PL D on PC, with the subsequent formation of DG by PA phosphohydrolase. Cabot et al (1988) using a rat embryo cell line REF52 and vascular smooth muscle cells suggested that vasopressin-induced PA formation was due to the action of a PtdIns-specific PL D. A PL D mechanism for the production of PA has also been proposed for the action of carbachol, histamine and substance P on guinea pig small

intestine longitudinal smooth muscle and that of A23187 on neutrophils, since no correlation was seen between PA formation and PPI turnover in either of these studies (Mallows and Bolton 1987; Balsinde et al 1988). PC hydrolysis has also been attributed to the action of a PC-specific PL C in vasopressin-stimulated rat hepatocytes and purinergic-stimulated rat liver plasma membranes (Besterman et al 1986; Irving and Exton 1987).

In Chapter 4 [<sup>3</sup>H]AA labelling was used as a means of determining PA production. Phosphoinositides are particularly enriched in AA and so this method enabled PA production from predominantly phosphoinositides to be studied. In view of the alternative sources of PA outlined above other methods for the determination of PA from other phospholipids would need to be utilised to study overall PA production. Cabot et al (1988) have used the preferential [<sup>3</sup>H]myristic acid labelling of PC to look at PA formation from this phospholipid and a sensitive HPLC and fluorimetric detection method was used to determine PA levels by Yamada et al (1988).

The relative importance of these two pathways for PA production, that using PL C and that employing PL D, may be dependent on the cell type and agonist under investigation. PA may play an important role in the early stages of stimulus-response coupling, for example, PA from PC hydrolysis, as well as phosphoinositides, may act on PL C to stimulate PPI breakdown. Cell-specific responses to a particular agonist may therefore reflect the ability of a

particular agonist receptor to interact with a certain phospholipase enzyme. The fatty acid and polar headgroup composition of the membrane phospholipids in the region of the agonist receptor may also influence which particular pathway is utilised. The possibility of PA being produced from different phospholipid sources also implies that DG of differing fatty acid compositions could be produced which could activate the different PKC isoenzymes that have been identified (Nishizuka 1988). The intracellular role of the phospholipid PA warrants further investigation in view of the emerging complex interactions of agonist-induced phospholipid cycles.

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